UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

Instituto de Biotecnología

Mutagénesis de residuos involucrados en la estabilidad al pH alcalino de la Penicilino acilasa de *E. coli.*

Tesis

que para obtener el grado de Maestro en Biotecnología

presenta:

el Ingeniero Gabriel Del Rio Guerra

Cuernavaca, Morelos Marzo de 1994

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AGRADECIMIENTOS

Un trabajo que resume mis últimos dos años de vida, y por lo tanto la mayor parte de la misma, no puede quedar completo sin que se mencionen a los protagonistas principales. Asi que, mas que un agradeciemiento, es un reconocimiento que quiero hacer público a través de este trabajo, a la enorme paciencia, y en algunos casos, cariño y comprensión, que mostraron hacia mi persona. La lista empieza asi:

A mis padres, Eustolio Del Rio Tapia e Hilda Esther Guerra Navarro.

A mis hermanos, Heberto, Vianey y Roxana Del Rio Guerra

A Susana Castro Obregón y su familia

A toda la gran familia "Culichi": Amalia Guerra Navarro, Jesusa Guerra Navarro, los Ernestos, Ramones, Alba, Perla, Imelda y sus acompañantes, los Victores, María Elena, Ramses y Cintia, los Juanes, Amalia, Uladimir, los Cuahutemoc, etc.

A todos ellos, por el cariño, paciencia, comprensión y todas las cualidades que se requieren para querer a un ser humano como yo, de la forma en que lo han hecho ellos.

A los miembros del Laboratorio de Xavier Soberón Mainero, incluyéndolo: Hector Viadiú Ilarraza, Joel Osuna Quintero, María Elena, Humberto, Ernesto, Marina, Gabriel, Francisco, Paul, Eugenio y Doña Juanita.

A ellos, por ayudarme a entender y a tratar a la gente y a los proyectos de investigación.

A los miembros de los laboratorios de Luis Covarrubias y Mario Zurita, incluyéndolos: Diana, Enrique, Luke y Jesus.

A ellos, por su comprensión y apoyo.

A los miembros del Instituto de Biotecnología y del Instituto Tecnológico de Sonora y a los Doctores Agustín López-Munguía y Francisco Lara Ochoa.

A ellos, por haber contribuído invaluablemente en mi formación académica con una aún incomprensible para mi, paciencia y en algunos casos, hasta dedicación.

Finalmente, a "Peque" y "Popis", por haber despertado en mi el sentimentalismo.

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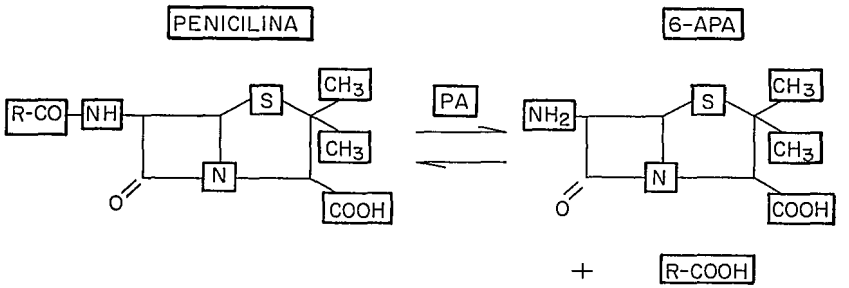
2.- Del Rio G., Rodriguez M.E., Munguía M.E., Lopez-Munguía A., and Soberón X., Mutant *E. coli* penicillin acylase with enhanced stability at alkaline pH. Sometida a publicación a la revista Biotechnology and Bioengineering.

I. INTRODUCCION

I.1 GENERALIDADES DE LA PAEC

La enzima Penicilino Acilasa (Penicilino amidohidrolasa; EC 3.5.1.11; PA) cataliza la hidrólisis de Penicilinas produciéndose el ácido 6-amino penicilánico (6-APA) y un ácido orgánico de acuerdo a la siguiente reacción:

Reacción 1.



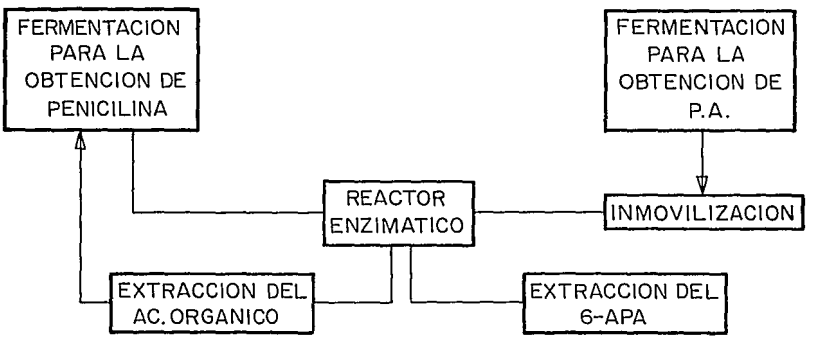
La naturaleza del ácido orgánico (R-COOH) depende del tipo de penicilina hidrolizada; el ácido fenil acético se produce cuando la bencil penicilina (Penicilina G) se hidroliza, mientras el ácido fenoxiacético se obteniene en la hidrólisis de la penicilina V. Esta reacción puede llevarse a cabo en sentido inverso al de la hidrólisis a valores de pH ácidos y las PAs catalizan esta reacción (1, 2).

Los organismos que producen PAs incluyen bacterias y hongos, y cada enzima obtenida presenta propiedades distintas, dependiendo de la fuente; por ejemplo, distintas PAs difieren en su especificidad a diferentes moléculas de Penicilinas (con distinto grupo sustituyente R-CO-), y se han clasificado de acuerdo a estas diferencias como Tipo I a las que preferencialmente hidrolizan Penicilina G, Tipo II para aquellas con preferencia a hidrolizar Penicilina V y Tipo III a las que pueden hidrolizar Ampicilina (3).

Las propiedades farmacológicas de la molécula de penicilina reside en el grupo sustituyente R-CO-, por lo que la funcionalidad de las PAs es importante para obtener, por una parte, al 6-APA que puede utilizarse como precursor de nuevos antibióticos, y por otra, llevar a cabo la síntesis de estos antibióticos asi como de otros compuestos (4, 5, 6, 7, 8).

I.2 BIOCATALISIS PARA LA OBTENCION DEL 6-APA

El proceso de obtención del 6-APA via enzimática, se muestra en el siguiente diagrama (9):



En este diagrama es importante destacar que, desde un punto de vista biotecnológico, existen varios pasos que pueden mejorarse: cepas, inmovilización de la enzima, formulación del biocatalizador, la enzima misma, etc.. En esta última etapa participó el laboratorio de Ingeniería de Proteínas, buscando mejorar la estabilidad de la enzima, en un proyecto general de mejoramiento de producción del 6-APA, que emplea a la PA de *E. coli* (PAEC).

Un problema inherente a la reacción es el control del pH. Durante la acción de la PAEC sobre la Penicilina G el medio se acidifica (véase Reacción 1); esto disminuye la eficiencia de conversión de Penicilina a 6-APA debido a que la reacción inversa se favorece y la vida media tanto de la enzima como del sustrato (penicilina G) disminuyen. Para mantener el pH a las condiciones adecuadas de la reacción, es necesaria la adición de

álcali, lo cual expone tanto al sustrato como a la enzima, a condiciones que reducen notablemente su vida media; estas adiciones de álcali finalmente reducen el rendimiento del bioreactor (10).

I.3 INGENIERIA DE PROTEINAS

La Ingeniería de Proteínas tiene como uno de sus objetivos el diseño de proteínas con funcionalidades deseadas. Para lograr esto se requiere de modificar a la proteína en su estructura primaria, lo que ha venido llevándose a cabo de dos formas (11, 12):

1) Alteración directa de la proteína.

2) Modificación Indirecta, através del gene que codifica para la proteína.

Aunque cualquiera de estos enfoques puede, en principio, lograr cambios funcionales en las proteínas, la modificación através del ADN resulta más conveniente para realizar Ingeniería de Proteínas, por su mayor versatilidad en la manera de introducir los cambios en éstas.

Además, existen diversas metodologías agrupadas en el inciso 2), las cuales a su vez pueden agruparse en:

1) Mutagénesis generalizada, es decir que se realiza sobre todo el gene.

2) Mutagénesis sitio dirigida, que se efectúa sobre una porción del gene.

Con la mutagénesis sitio dirigida es posible diseñar cuales y cuantos cambios se introducirán en una porción deseada del gene e incluso a varias porciones en el mismo gene. La importancia de esto último radica en que la funcionalidad de una proteína no esta distribuída a lo largo de toda su estructura primaria, si no que se localiza en solo algunos residuos de aminoácido, que generalmente no se encuentran contínuos en la estructura primaria; además, no siempre es posible predecir cual es el cambio o los cambios necesarios para lograr la función deseada, por lo que también es necesario sustituir por diversos aminoácidos cada uno de los residuos involucrados en la función.

Una de las metodologías que permite realizar, con este detalle y reproductibilidad las modificaciones en las proteínas, es la de la Reacción de la Polimerasa (PCR)(13). Esta requiere de un par de oligonucleótidos, la polimerasa y el ADN a modificar (véase Métodos).

Una vez generada la variabilidad deseada en una población de mutantes de la enzima en estudio, es necesario seleccionar a aquellas con la funcionaliad deseada; esto impone una restricción en el número de variantes a ensayar. Si se supone que existen 10 residuos de aminoácido que están involucrados en la función a alterar y que no se sabe cuales son los cambios que deben realizarse, esto implica que se deben ensayar las 20^{10} posibles mutantes; para lograr esto, es necesario producir un banco de colonias bacterianas, generadoras de las proteínas mutantes, al menos igual en número a 20^{10}. Desafortunadamente, no existe aún ninguna metodología capaz de transformar bacterias con tal eficiencia. Por otra parte, es muy probable, de acuerdo con los resultados obtenidos en diversos sistemas, que proteínas que contengan un número limitado de reemplazos de aminoácidos, puedan mostrar importantes alteraciones en su función. Para reemplazos dobles, el número de mutantes a ensayar es de 18,000 (20*20*[10!/(8!*2!)]) y aunque metodológicamente, aún no es posible producir un banco de mutantes dobles exclusivamente, si es posible crear uno en donde se encuentren representadas todas las mutantes dobles y en mayor proporción que cualquier otro tipo de mutantes. El siguiente problema a resolver es como producir ese banco y de que tamaño debe de ser, y asi determinar la posibilidad de encontrar la mutante deseada. Los resultados de el análisis de este problema pueden ayudar a conducir experimentos de mutagénesis de manera racional (véase Trabajos Sometidos a Publicación No. 1).

Ya que la estructura tridimensional de la PAEC aún no se conoce (14), no es posible aún realizar un diseño adecuado de mutaciones. Es necesario entonces emplear un método azaroso de mutagénesis y un método de ensayo. El metodo de ensayo que escogimos se basa en la reacción colorida que se da al reaccionar el p-dimetilamino-benzaldehído (PDAB) con el 6-APA. El PDAB genera un compuesto colorido al reaccionar con el grupo amino libre que queda en el 6-APA después de la hidrólisis de la penicilina por PAs (Véase Métodos para la implementación del método).

Es evidente que el conocer cuales residuos de aminoácido están involucrados en la función de estudio, puede mejorar el diseño para obtener la nueva función, pero el empleo de un método de ensayo apropiado permite mutagenizar extensamente el gene en cuestión. Esta mutagénesis puede ser generalizada o sitio dirigida; para esta última es necesario determinar los posibles residuos de aminoácido involucrados en la estabilidad mediante el empleo de métodos de predicción de estructura. Sin embargo, la alteración de funcionalidades en las proteínas por predicciones, dependen de una caracterización exahustiva (15, 16, 17, 18) y tal no es el caso de las PAs, por lo que existe un grado de incertidumbre cuando se mutageniza de manera sitio dirigida en estas condiciones.

I.4 ESTABILIZACION DE PROTEINAS

La modificación de la estabilidad de proteínas está bien documentada e incluso se han propuesto reglas sobre como lograr esto mediante Ingeniería de Proteínas (19, 20, 21), las cuales se sustentan en la hipóteisis de que hay ciertas interacciones en la proteína que favorecen la conformación funcional. Otras alternativas también empleadas son:

1) Exposición a solventes orgánicos (5, 22).
2) Inmovilización (23, 24).

Por otra parte, la modificación de la estabilidad de proteínas hacia pHs determinados requiere de otras consideraciones (25, 26, 27). Por ejemplo, se ha observado que el pK de los residuos involucrados en la catálisis enzimática, es un factor determinante para la eficiencia de la reacción (28). Además, la alteración de residuos de aminoácidos con carga, lejanos al sitio activo, pueden llevar a modificar el pH óptimo de actividad y estabilidad (27). Una observación que ha conducido a explicar este último fenómeno, es la relación directa que guardan el punto isoeléctrico (PI) y el pH óptimo de estabilidad en las enzimas. Lo que se postula actualmente es que la repulsión ocasionada entre cargas de igual signo, en la superficie de la proteína, propicia la pérdida de la conformación funcional en las enzimas; esta repulsión no ocurrirá en rangos de pH donde los residuos de aminoácido no tengan carga, lo cual sucede alrededor del PI (25).

Es importante considerar que aunque es necesario un plegamiento estable para que las enzimas puedan realizar su función, el mecanismo de catálisis impone restricciones en los valores de pH del microambiente del sitio activo y que por lo tanto, es muy interesante la correlación observada entre PI y pH óptimos de estabilidad. Adicionalmente, el caso de las PAs de *E. coli* y de *Kluyvera cytrophila* (PAKC) (enzimas 85 % homólogas en su estructura primaria), es un ejemplo de lo expuesto anteriormente: ambas poseen un PI y pH óptimo de estabilidad = 6, sin embargo, la PAKC es mas estable a pH alcalinos (29).

Se puede mejorar el PI de una enzima mediante Ingeniería de Proteínas, al eliminar o introducir cargas, particularmente en el exterior de la enzima. La alteración de cargas en el interior de las proteínas puede llegar a desfavorecer el estado nativo (funcional) de éstas o bien el pK de residuos involucrados en catálisis, lo que generalmente reduce la actividad enzimática.

I.5 CARACTERISTICAS RELEVANTES DE LA PAEC PARA SU ESTABILIZACION

De las PAs, la mas estudiada es la PAEC, sin embargo, su caracterización estructural apenas empieza. Se desconoce su estructura tridimensional sola o en complejo y los estudios en Ingeniería de proteínas, hasta la fecha, son muy limitados (30, 31, 32, 33). Los datos sobre PAEC relevantes al proyecto son:

1) Consta de 2 subunidades, una denominada alfa, que tiene 209 residuos de aminoácido y otra denominada beta, que posee 557 residuos (34).

2) Esta enzima pasa por un proceso de maduración pos-traduccional; se traduce como una pre-pro-proteína; el pre-péptido, o péptido lider, consta de 25 aminoácidos y sirve para conducir a esta enzima al espacio periplásmico, en donde el pro-pétido o péptido de unión (40 aminoácidos) entre las subunidades, es procesado obteniéndose así, la enzima en su forma activa (35).

3) Experimentos de plegamiento *in vitro,* muestran que para que se forme una enzima funcional a partir de las subunidad alfa y beta, es necesario que la subunidad alfa esté plegada primero que la subunidad beta (36).

4) Se ha observado que la actividad *in vivo* de PAEC es termoregulada (37) lo que se ha atribuido a la termosensibilidad de la subunidad alfa (36) y al prerequisito de su plegamiento en la constitución de la enzima funcional (35, 36, 38, 39).

5) El sitio de reconocimiento del ácido orgánico en la PAEC, es decir, el componente principal de la especificidad, aparentemente está ubicado en la subunidad alfa (30, 40).

6) Se ha propuesto que la porción amino terminal de la subunidad alfa es la responsable de la interacción con la subunidad beta (30).

7) Se postula que el mecanismo de acción de las PAs es semejante al de las Serin-proteasas por la sensibilidad compartida al Fenil Metil Sulfonil-Fluoro (FMSF), y el tipo de enlace sobre el que actuán (enlace amídico) (2). En el mecanismo catalítico de las Serin-proteasas están involucrados tres aminoácidos (comúnmente denominados "triada catalítica"): Serina, Histidina y Aspártico, siendo la Serina la que lleva a cabo el ataque nucleofílico sobre el carbonilo del enlace amídico. Los estudios de inactivación por FMSF señalan a la subunidad beta como la portadora de esta Serina catalítica (41).

8) Experimentos *in vitro* mostraron que la subunidad beta se desnaturaliza al variar el valor del pH (36).

9) La deleción de la porción carboxilo terminal de la subunidad beta impide que la asociación de las dos subunidades sea productiva (36).
10) Están reportadas solamente las secuencias de PAEC, PAKC, una PA que degrada preferentemente Penicilina V (a diferencia de PAEC y PAKC que degradan preferentemente Penicilina G) y tres Cefalosporino acilasas (Información obtenida de la base de datos GenBank).

11) La modificación química y sitio dirigida de Serinas en PAEC ha involucrado a la Serina 290 (S290), de la subunidad beta, en el mecanismo

catalítico de esta enzima. (41, 42)

12) La modificación de los residuos de aminoácido Lisina 374 (K374), Histidina 481 (H481) y Metionina 168 (M168) en PAKC, ha mostrado que éstos juegan un papel importante en la catálisis (30, 33).

I.6 ANTECEDENTES

Para la realización de este proyecto se partió de los datos ya mencionados y de la experiencia lograda en este Instituto en cuanto a diferentes aspectos de la PAEC, como lo son (10, 43):

1) Clonación y sobre expresión del gene en un vector para *E. coli*.

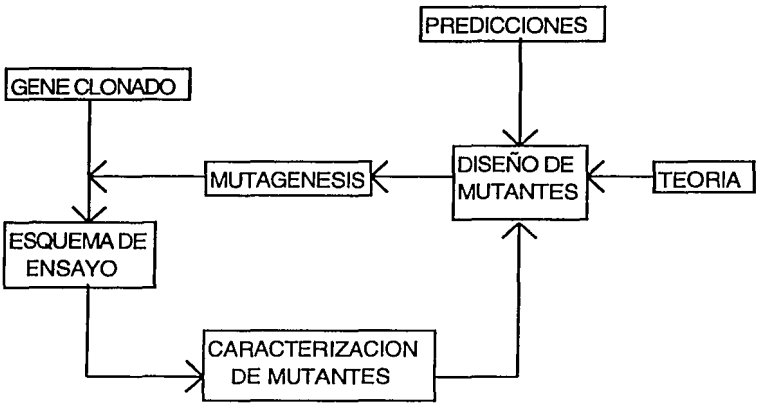
2) Caracterización de la regulación en la sobreproducción.

3) Caracterización de las condiciones de operación en un reactor.

Ya que la estabilidad al pH el problema a resolver en este estudio, es importante destacar que la PAEC inmovilizada tiene un pH óptimo de estabilidad de 7.5, y que la pendiente observada de pérdida de actividad para valores de pH mayores a éste, es más pronunciada que para valores menores de pH 7.5 (10). Debe tenerse en cuenta que a pH ácidos (pH 5-6) la reacción de síntesis se favorece, por lo que las determinaciones hechas para determinar la estabilidad a pH menores a 7.5 pueden ser el reflejo de la suma de la inestabilidad y la reducción en la tasa de actividad hidrolítica. Esto indica que la PAEC puede ser mas sensible pH alcalinos, por lo que es en ésta característica donde resultados más relevantes para la producción del 6-APA podría obtenerse.

II. ESTRATEGIA

El problema que se busca resolver en este estudio es el de la estabilidad a pH mayores a 7.5 de la PAEC y para ello se planteó la siguiente estrategia:



El fundamento de esta estrategia es que mediante la alteración de residuos de aminoácidos localizados en la superficie de la PAEC, es posible modificar el PI de la misma y con ello se espera que también se modifique el pH óptimo de estabilidad. Ya que lo se pretende es mejorar la estabilidad a pH alcalinos (> 7.5), la mutagénesis que debiera lograr esto es aquella que introduciría aminoácidos con pK mayores a 7.5, es decir, se espera que al aumentar el número de este tipo de residuos en la superficie de la enzima, se requiera aumentar el valor del pH en el medio para neutralizar estas cargas, y asi lograr un plegado estable de la PAEC.

En resumen, la estrategia requiere de establecer un método de ensayo. Una vez logrado esto, hay que proceder a mutagenizar el gene de la PAEC. La mutagenésis podrá aplicarse con un grado de variabilidad controlada y para ello, será necesario restringirla a residuos de aminoácido que pudieran estar involucrados en la estabilidad al pH, mismos que se pueden seleccionar a partir de un estudio predictivo de la topología de PAEC, para lo que se emplearán predicciones de estructura

secundaria, análisis de hidropaticidad, homología en secuencia y datos bioquímicos sobre las PAs. Considerando que la predicciones no son del todo confiables, sobre todo para un sistema como el de PAEC, es conveniente emplear también una mutagénesis generalizada. Finalmente, las PAECs mutantes generadas por estas estrategias de mutagenesis deberán ser evaluadas con el método de ensayo establecido y asi aislarlas para su ulterior caracterización.

III. OBJETIVOS

III.1 OBJETIVO GENERAL

Obtener mutantes de PAEC con estabilidad mejorada a pH alcalino (>7.5).

III.2 OBJETIVOS PARTICULARES

1) Implementar un método de ensayo que permita aislar mutantes de estabilidad.

2) Crear banco de mutantes de PAEC de tamaño adecuado para rastrearse con el método de ensayo.

3) Estabecer las condiciones necesarias para realizar mutagénesis combinatoria sitio dirigida por PCR, sobre el gene de la PAEC.

IV. METODOS

IV.1 METODO DE SELECCION

IV.1.1 REACTIVOS

a) Penicilina G: 200 mg/mL de solución amortiguadora de fosfatos, pH 7.8.

b) Paradimetil Amino Benzaldehído: Solubilizar 1 gramo de paradimetil en 50 mL de etanol al 60% (con agua v/v). Agitar y agregar 0.5 mL de ácido sulfúrico concentrado y aforar a 100 mL con etanol al 60%.

c) Soluciones amortiguadoras: Todas con molaridad 0.1 M; pH 5 citratos; pH 7.8 fosfatos de potasio; pH 10 glicina/NaOH.

IV.1.2 METODOLOGIA

a) Crecer en caja petri, con medio Luria y kanamicina, 12 horas a 37 grados centígrados, células de *E. coli* portadoras de PAEC.

b) Picar con mondadientes esterilizados las colonias crecidas e incubar nuevamente 50 colonias por caja petri. Cada colonia del inciso a) se reproducía por triplicado para ensayar a pH de 5, 7.8 y 10.

c) Transferir a papel Whatman 540.

d) Exponer a vapores de cloroformo durante 30 minutos; para ello empleamos un desecador que contenía en su interior un recipiente abierto con cloroformo líquido. Con esto se lisaba la pared celular y asi quedaban expuestas las proteínas periplásmicas que entonces se adhieren al papel.

e) Sumergir en recipientes con soluciónes amortiguadoras a los distintos pH a ensayar, cada uno de los papeles Whatman obtenidos del inciso d). El tiempo de exposición fué de 1 y 3 horas.

f) Sacar los papeles y lavar 3 veces con solución amortiguadora pH 7.8.

g) Bañar con solución de penicilina G e incubar a 37 grados centígrados durante 2 horas o hasta sequedad.

h) Bañar con solución de paradimetil y observar coloración.

IV.2 MUTAGENESIS

IV.2.1 MUTAGENESIS QUIMICA

IV.2.1.1 REACTIVOS

a) Solución 1: Hidroxilamina 2M (P.M. 69.5) disuelta en etilenglicol. Esta solución debe prepararse justo antes de usarse o deberán guardarse alicuotas en congelación.

b) Solución 2: Solución amortiguadora, 70mM de fosfato de potasio, 0.6 M de acetato de sodio, pH 5.2

c) Solución 3: Solución amortiguadora de acetato de sodio 3M, pH 5.2

IV.2.1.2 METODOLOGIA

a) Adicionar 5 microgramos de plásmido (pBGS19PAC) a 50 microlitros de solución 2.

b) Adicionar 50 microlitros de la solución 1, mezclar e incubar a 65 grados centígrados.

c) Remover 25 microlitros a los 0, 1, 2, 3 ... 15 minutos e inmediatamente depositarlos en tubos de microcentrífuga que contienen 25 microlitros de agua, 5 microlitros de solución 3 y 125 microlitros de etanol absoluto frío. Reposar 10 minutos en hielo y centrifugar por 10 minutos. Remover el sobrenadante y lavarlo con 400 microlitros de etanol 70% frío; centrifugar 1 minuto, remover el sobrenadante y secar el precipitado.

d) Transformar células competentes de *E. coli* por electroporación (48).

e) Aplicar el método de ensayo.

IV.2.2 MUTAGENESIS SITIO DIRIGIDA

IV.2.2.1 MATERIALES

a) Células competentes: Se prepararon de acuerdo a (48).

b) Electroporador: Marca Bio-Rad, modelo 1652075.

c) Plásmidos: pBGS18PAC y pBGS19PAC obtenidos a partir de pBGS18 y 19 (49) respectivamente. El pBGS19PAC fué cedido por el Dr. Enrique Merino (43) y el pBGS18PAC fué construido a partir de la subclonación del gene de la PAEC contenida en el pBGS19PAC, mediante los sitios de Eco RI y Hind III (véase fig. 1).

d) Oligonucleótidos mutagénicos: Estos fueron sintetizados en la Unidad de Síntesis Molecular del Instituto de Biotecnología/UNAM. Las letras minúsculas representan los codones mutagenizados de acuerdo con la siguiente estrategia:

La mutagénesis por los oligos se produce porque lo que se sintetiza en realidad es una familia de oligos que se diferencian en secuencia. Esta diferencia se logra cuando al sintetizarlos, en la primera posición del codón a mutar, se añade Adenina (A) y Citosina (C), en la segunda posición A y Guanina (G) y en la tercera posición A, Timina (T), G y C. Esta mezcla, en concentraciones adecuadas, debe producir un espectro de 6 aminoácidos (véase tabla 1): Lisina (4/36), Arginina (10/36), Histidina (8/36), Asparagina (4/36), Glutamina (8/36) y Serina (2/36). Además, durante la síntesis se incluyó las bases del codon sin alterar en un 25% del total, de esta forma se produce una distribución de oligonucleótidos mutantes en la que se representa, en mayor número, aquellos que introducen en su secuencia, dos codones mutados (Véase Trabajos en publicación para una explicación mas detallada sobre este último aspecto de la síntesis).

A continuación se describen los olinucleótidos empleados para introducir las mutaciones en el gene de PAEC, y los complementarios para extender todo el gene. Las letras en minúsculas representan los codones seleccionados a mutagenizar, y en la Tabla1 se presentan los amino ácidos (con sus correspondientes codones) generados en cada codon mutado.

Oligo 1 (Subunidad beta):
5'-GGCTGCGCTGgaagatACCTGGgagACTCTTTCCAAACGC-3'

Oligo 2 (Subunidad beta):
5'-GGTTATGTTCATactGGTgctTATCCAgatCGTCAATCAGGC-3'

Oligo 3 (Subunidad alfa):
5'-AACGGCTTTAAAAgatAAATATGGTgtatcacaaGGCATGGCGG-3'

Oligonucleótidos extensores:

Oligo 4 (oligo universal para secuenciar en M13):
5'-ACTGGCCGTCGTTTTACAAC-3'

Oligo 5 (oligo reverso):
5'-GGAATAGCCGATCGAGATCTGTAACCAGCCCTCCA-3'

Oligo 6 (oligo tapón):
5'-GGAATAGCCGATCGAGATCT-3'

TABLA 1. DISTRIBUCION Y CARACTERISTICAS DE AMINOACIDOS OBTENIDOS CON LA ESTRATEGIA DE MUTAGENESIS.-

CODON	AMINO ACIDO	CARGA	pK
AAA/G	Lisina	(+)	10.5
AAT/C	Asparagina	(+/-)	-
AGA/G	Arginina	(+)	12.5
AGT/C	Serina	(+/-)	-
CAA/G	Glutamina	(+/-)	-
CAT/C	Histidina	(+)	6.0
CGA/T	Arginina	(+)	12.5
CGG/C	Arginina	(+)	12.5

e) Reactivos para PCR: Fueron empleados los distribuidos por Biolabs para Taq polimerasa.

IV.2.2.2 METODOLOGIA

a) Técnica de la Reacción de la Polimerasa (PCR) (13):

Esta técnica requiere de un ADN como templado (pBGS18PAC), un par de oligos (30 picomolas de cada uno), enzima y solución amortiguadora. En todos los casos las reacciones se realizaron en 0.1mL de volumen final. Para lograr producir el ADN a partir de esto se aplican 20 ciclos y durante cada ciclo tres temperaturas : para desnaturalización (92 grados centígrados), para recocido (55 grados centígrados) y para extensión (70 grados centígrados). Cuando se empleó algún oligo mutagénico, se realizó un ciclo previo a los 20 con una temperatura para recocido de 50 grados.

La mutagénesis fué diseñada para introducir mutaciones en tres zonas predichas como expuestas al disolvente, una en la subunidad alfa y

dos en la beta. 6 oligonucleótidos fueron empleados para ello. Los oligos 4, 5 y 6 fueron diseñados basados en las secuencias adyacentes a los sitios múltiples de clonación en los vectores de las series M13mp y PUC (4 y 5 son en realidad extensiones de los oligos universal y reverso empleados para secuenciación) y deben ser útiles para cualquier ADN clonado en ellos. Los oligos 1, 2 y 3 introducen las mutaciones. En una primera reacción se utilizó a los oligos 1 y 5 bajo las condiciones descritas y se obtuvo el producto PCR I (Véase ref. 13) el cual fué purificado de gel de agarosa al 0.8 %, y 100 nanogramos de éste fueron empleados junto con el templado (pBGS18PAC sin digerir) para su extensión; después del primer ciclo a 50 grados se añadieron los oligos 2 y 6. Nótese que el oligo 6 no es complementario al templado y entonces, debe solamente amplificar ADN derivado de la extensión del producto I; asi se obtiene el producto de PCR II. Finalmente, este producto de PCR y los siguientes fueron empleados como el I utilizando los oligos 3 y 4 sucesivamente para amplificar todo el gene de PAEC con las tres zonas mutadas simultánea y combinatoriamente.

b) Clonación y expresión de los productos de PCR:

La clonación se realizó de dos formas; una clonando todo el fragmento correspondiente al gene PAEC con las tres zonas mutadas (fragmento EcoRI-HindIII), y otra clonando por separado las subunidades (subunidad β: EcoRI-BglII, subunidad α: BglII-HindIII). Para las clonaciones se empleó al vector pBGS19 (gene completo) y pBGS19PAC (clonación de subunidades), para que de esta forma se tradujera la hebra codificante del gene PAEC (véase figura 1).

Para expresar las proteínas mutadas de PAEC ya clonadas, se utilizó la maquinaria de *E. coli*. Para ello hubo que transformar las células de esta bacteria con los plásmidos portadores de los genes mutantes. La metodología de transformación que nos permitiría analizar el mayor número de plásmidos mutantes es la de electroporación (48).

IV.3 PREDICCIONES DE TOPOLOGIA EN PAEC

IV.3.1 HOMOLOGIA DE SECUENCIA

Para ello se aplicó el algoritmo de LFASTA (47), el cual alinea pares de secuencias. Para generar el alineamiento múltiple con las dos penicilino acilasas y las tres cefalosporino acilasas, se alinearon todas contra la PAEC, subunidad por subunidad, y posteriormente se alineó manualmente a todas, tomando como referencia a la PAEC. Los parámetros empleados fueron los estándar.

IV.3.2 ESTRUCTURA SECUNDARIA

IV.3.2.1 ENFOQUE DE CHU-FASMAN (44)

Los parámetros empleados para este enfoque fueron los estándar para proteínas globulares.

IV.3.2.2 ENFOQUE DE GARNIER (45)

El algoritmo empleado utiliza la versión II de este enfoque (45). Los parámetros utilizados hacían corresponder los porcentajes de α-hélices y β-plegadas predichos para cada subunidad de la PAEC y PACKC con los experimentalmente determinados respectivamente (50, 51). Como estos porcentajes son muy parecidos se promediaron y esos fueron los empleados para las cinco secuencias analizadas.

IV.3.2.3 PATRON DE HIDROPATICIDAD

Los parámetros utilizados fueron los reportados por Kyte y Doolittle (46).

IV.4 AISLAMIENTO DE MUTANTES

Una vez observado el fenotipo con el método de ensayo, aislamos el

ADN correspondiente. Esto se realizó de la siguiente manera:

1) Transformamos cepas con el gene mutante tres veces consecutivas, es decir, se purificó el plásmido de cada transformación y este se empleó para transformar otra bacteria; a esta bacteria transformada se le verificó el fenotipo y se aisló su ADN. Este procedimiento se repitió tres veces.

2) Transferencia del fenotipo a un gene silvestre de PAEC por subclonación de fragmentos. Esto se realizó de acuerdo a la subunidad que se mutagenizara (Véase fig. 1).

IV.5 SECUENCIACION DE MUTANTES

Para secuenciar los fragmentos portadores de las mutaciones se utiliaron dos enfoques: por PCR (13) o bien por Sequenasa (Kit de USB); de esta última se probó tanto para doble cadena como para cadena sencilla. Para ello se sintetizaron 3 oligonucleótidos a 60 bases de las sitios mutagenizados:

Oligo 1 (subunidad alfa):
5'-GCGCTGGGAACCGTTTGATG-3'

Oligo 2 (subunidad beta):
5'-GGCAGGAGTGGACACAGCAG-3'

Oligo 3 (subunidad beta):
5'-TGAGGCGGTGCAGGGAGACA-3'

Adicionalmente se emplearon los oligos universal y reverso.

Ulteriormente fué necesaria la síntesis de 3 oligonucleótidos para secuenciar el fragmento BglII/HindIII de la mutante MI (Véase fig. 1):

oligo 4:
5'-CGGGATATATTGCTAACTGG-3'

oligo 5:

5'-AGAGCGATCCGCGTCGTCAG-3'

oligo 6:
5'-CACCTGCAATGGCCTTAACG-3'

V. RESULTADOS Y DISCUSION

V.1 METODO DE ENSAYO

De los ensayos realizados para determinar las condiciones de pH y tiempo de exposición necesarios, para que las células portadoras de la PAEC no dieran coloración en presencia del PDAF, se obtuvieron los siguientes resultados al ensayar 50 colonias de *E. coli* portadoras del gene PAEC silvestre:

	pH		
	5.0	7.8	10.0
TIEMPO			
1 Hr.	+++	++	++
3 Hr.	++	+	-

+++: Halo amarillo perfectamente distinguible del transfondo.
++: Halo amarillo pálido distinguible del transfondo.
+: Halo difusamente distinguible del transfondo.
-: No aparición de halo.

En base a esto, se realizaron ensayos solo a 3 horas de exposición y a pH 10 con las mutantes producidas. Estos resultados concuerdan con los determinados para la enzima purificada, es decir, la mayor estabilidad se presenta a pH ácido (10).

V.2 PREDICCION DE TOPOLOGIA EN PAEC

Véase Trabajos Sometidos a Publicación No. 2.

V.3 MUTAGENESIS

V.3.1 MUTAGENESIS QUIMICA

Las mutantes obtenidas a partir de esta mutagénesis se sometieron al método de ensayo (3 Hrs. a pH 10.0) y de esta forma encontramos dos colonias que daban coloración bajo estas condiciones. Sin embargo, después de la segunda resiembra el fenotipo desapareció. No se dió mas seguimiento a esta técnica en vista de los resultados con mutagénesis sitio dirigida.

V.3.2 MUTAGENESIS SITIO DIRIGIDA

De los tres oligos mutagénicos diseñados, finalmente solo se pudo mutagenizar a partir de los llamados 1 y 3 ya que el 2 daba como producto mayoritario en la reacción de PCR, un oligonucleótido ligeramente de mayor peso molecular que el esperado, inclusive a partir de este producto de PCR no se pudo extender el gene completo de la PAEC.

A partir de la mutagénesis realizada sobre la subunidad beta con el oligo 1, se aisló una colonia con el fenotipo deseado. Esta mutante, denominada M1, se usó para transformar tanto a JM101, CMK y HB101. Finalmente se optó por mantener esta mutante en la cepa JM101 porque para las otras cepas este fenotipo era nocivo.

De la mutagénesis realizada con el oligo 3 en la subunidad alfa se aisló otra colonia más que presentaba el fenotipo buscado, y se le denominó M3. Después de aislarla con el mismo procedimiento que la anterior, se mantuvo en la cepa JM101.

En total, se ensayaron 1500 colonias (500 para las mutantes de la subunidad beta y 1000 para la alfa). Es interesante observar que, siendo la subunidad beta la que presenta una dependencia para su estabilidad al pH (50), sea en ésta en la que se tuvo que rastrear un menor número de mutantes.

Adicionalmente, aunque se pudo obtener un producto de PCR que combinara las mutantes de la subunidad α y β, en nuestras manos, no se pudo clonar este producto.

V.4 SECUENCIACION DE MUTANTES

La secuenciación de la mutante M1 reveló un cambio de Guanina a Citosina en el codon del Triptofano 431 generándose una Arginina (W431R). La secuenciación del fragmento BglII-EcoRI, portador de la mutación (Véase fig. 1 y Métodos), se logró con Sequenasa en cadena sencilla, para lo que se requirió subclonar el gene completo de la PAEC en Mp8 con Eco RI y Hind III (Véase fig. 1).

Al analizar la secuencia nucleotídica de la mutante M3 obtenida con los oligonucleótidos para secuenciar la subunidad alfa y beta, no se detectó ningún cambio. Para verificar la localización aproximada de esta mutación se subclonó el fragmento BglII-HindIII a pBGS19PAC (véase fig. 1) y se analizó el fenotipo observado con nuestro método de ensayo. De este ensayo se comprobó que en este fragmento se localiza(n) la(s) mutación(es) responsable(s) del fenotipo observado. La naturaleza de la(s) mutación(es) está siendo determinada.

V.5 CARACTERIZACION CINETICA

Los resultados de este trabajo están descritos en el artículo sometido a publicación 2. Cabe destacar que las constantes cinéticas y el perfil de pH de la mutante M1 fueron los mismos que la silvestre y que, el único efecto observado, fué la mejoría en la estabilidad a pH alcalino (pH= 8), a largo plazo, lo que era de esperarse si la mutación estuviera en la superficie de la enzima; es decir, de acuerdo con la hipótesis de que la distribución de las cargas en la superficie determina el pH en el cual las proteínas son mas estables, cabría esperar que la introducción de una carga positiva en la superficie desplazara el PI hacia pH mas alcalinos y con ello el pH óptimo de estabilidad, aunque no habría razón para esperar una alteración del perfil de pH ya que este esta determinado, principalmente, por los pK de los residuos involucrados en la catálisis.

La caracterización de la M3 mostró que tanto el perfil de pH (véase figura 2) como la estabilidad a largo plazo a pH ácido (pH=5 y 6) y alcalino (pH=8 y 10) (véase figura 3A, 3B, 3C y 3D) se alteraron con respecto a la silvestre. Ya que esta mutante es mas estable que la M1 y la silvestre a pH ácido, su utilización en la reacción de síntesis de antibióticos está siendo

estudiada en el laboratorio de Cinética enzimática.

Al comparar la secuencia de la PAEC con la PAKC, se observa que el Triptofano mutado en M1 se conserva en PAKC, por lo que es de esperarse que la estabilidad a pH alcalinos en esta última pueda mejorarse através de esta mutación. En este contexto, la mutante M1 tiende a parecerse a la PAKC, en lo que respecta a su estabilidad mejorada a pH alcalinos con respecto a PAEC. Esto podría ser una explicación de la diferencia observada en la estabilidad a pH alcalinos de PAEC y PAKC, es decir, esta última aparentemente posee un distribución de cargas positivas mayor que la PAEC. La verificación de esta hipótesis tendrá que basarse en una caracterización estructural de estas enzimas.

Un resultado desfavorable a las mutantes M1 y M3, es que las células portadoras de éstas, mostraron una baja actividad específica de PAs, por lo que se esta trabajando para resolver este problema en el laboratorio de Cinética enzimática.

VI. CONCLUSION

Se logró implementar y validar el método de ensayo necesario para aislar las variantes de PAEC con mayor estabilidad a pH alcalino. Una alternativa interesante de este ensayo es adaptarlo para seleccionar mutantes con mayor estabilidad a pH ácidos donde la reacción de síntesis se favorece y también la enzima silvestre es poco estable.

La mutación responsable del fenotipo observado en la mutante de la subunidad beta (M1) concuerda con lo predicho por la teoría, lo que implica que esta zona debe estar expuesta al disolvente.

La mutante M3 adquirió una mayor estabilidad, tanto a pH 6 como a pH 8, respecto a la enzima nativa y mutante M1; por ello se está estudiando su comportamiento en un bioreactor destinado a la síntesis de antibióticos. El fenotipo de esta mutante no corresponde con las predicciones realizadas; un intento de explicar el por qué ocurre este comportamiento deberá basarse en la determinación de la naturaleza de la(s) mutación(es) efectuada(s). La secuenciación completa de esta mutante está en espera de los resultados obtenidos en el biocatalizador.

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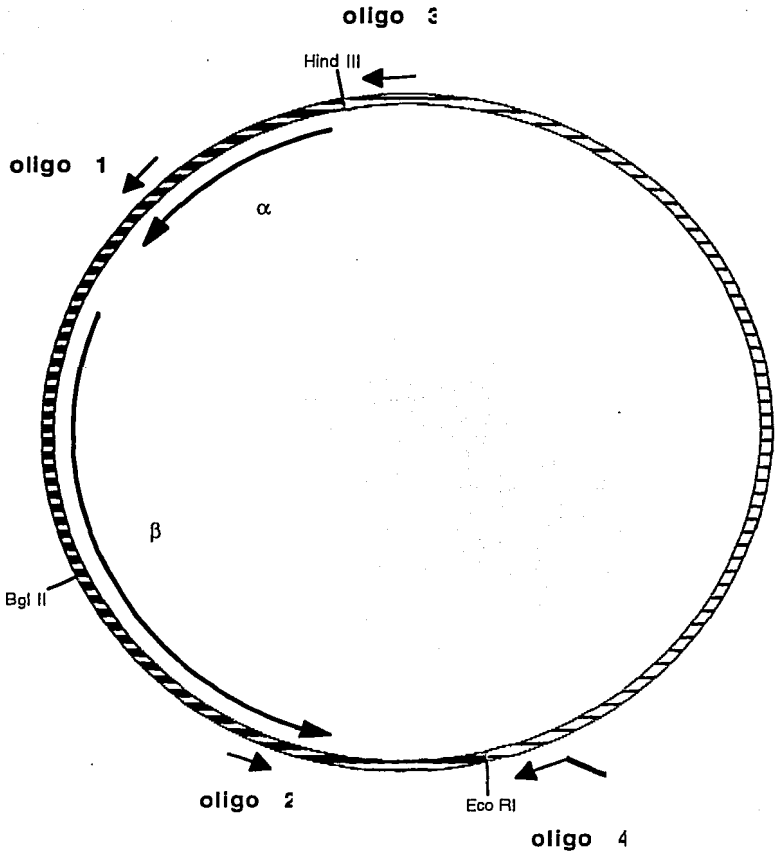
PIES DE FIGURA.

FIGURA 1.- Se muestran los sitios de restricción utilizados para la clonación y subclonación del gene PAEC (Eco RI y Hind III) y las subunidades de la PAEC α (Hind III-Bgl II) y β (Bgl II-Eco RI), respectivamente. A su vez, los oligonucleótidos utilizados para producir las mutantes M1 (PCR1) y M3 (PCR2). El plásmido pBGS18PAEC se obtuvo a partir del pBGS19PAEC (véase Métodos).

FIGURA 2.- En la gráfica se comparan los perfiles de actividad a distintos pH de la enzima silvestre y mutantes M1 y M3 (véase Trabajos en Publicación No. 2 para una explicación de la metodología empleada).

FIGURA 3.- Se presentan cuantitativamente las diferencias observadas por el método de ensayo en caja petri de las mutantes (M1 y M3) y la silvestre (PAEC), se determinaron las actividades residuales de cada una de ellas al paso del tiempo a pH 5 (3A), 6 (3B), 8 (3C) y 10 (3D) (véase Trabajos en Publicación No. 2 para una explicación de la metodología empleada)



pBGS18PAEC

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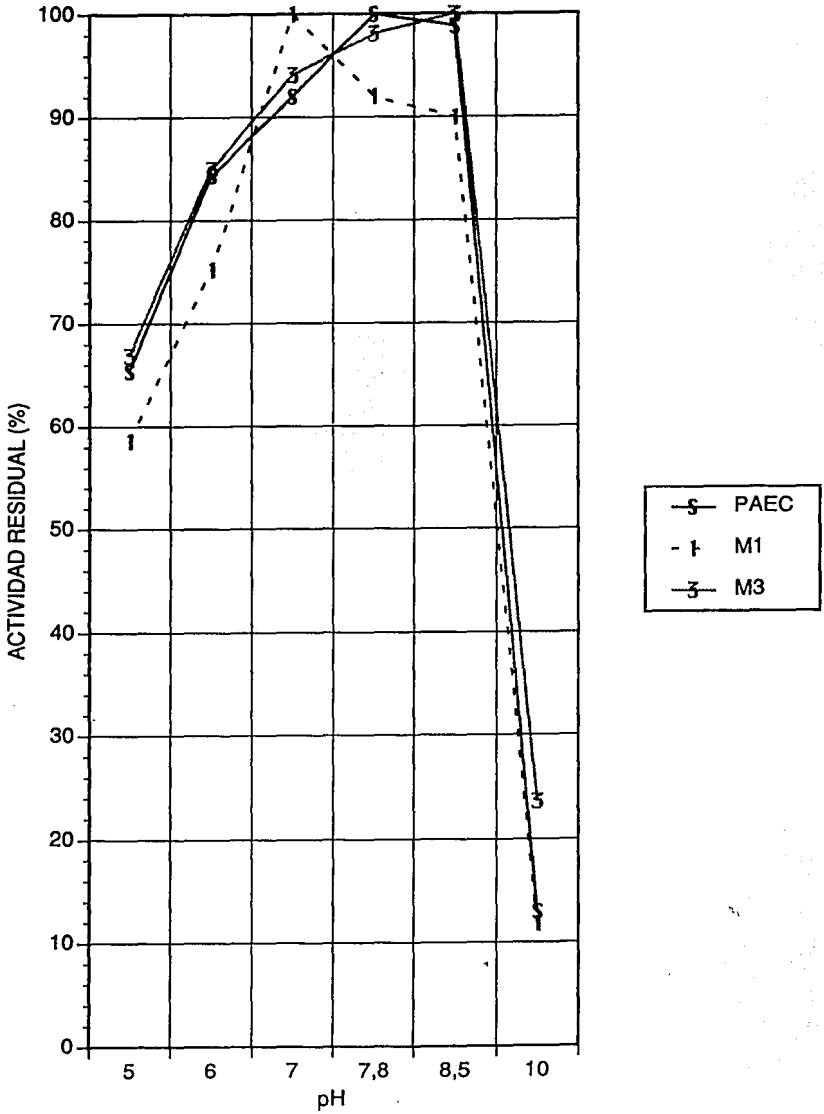
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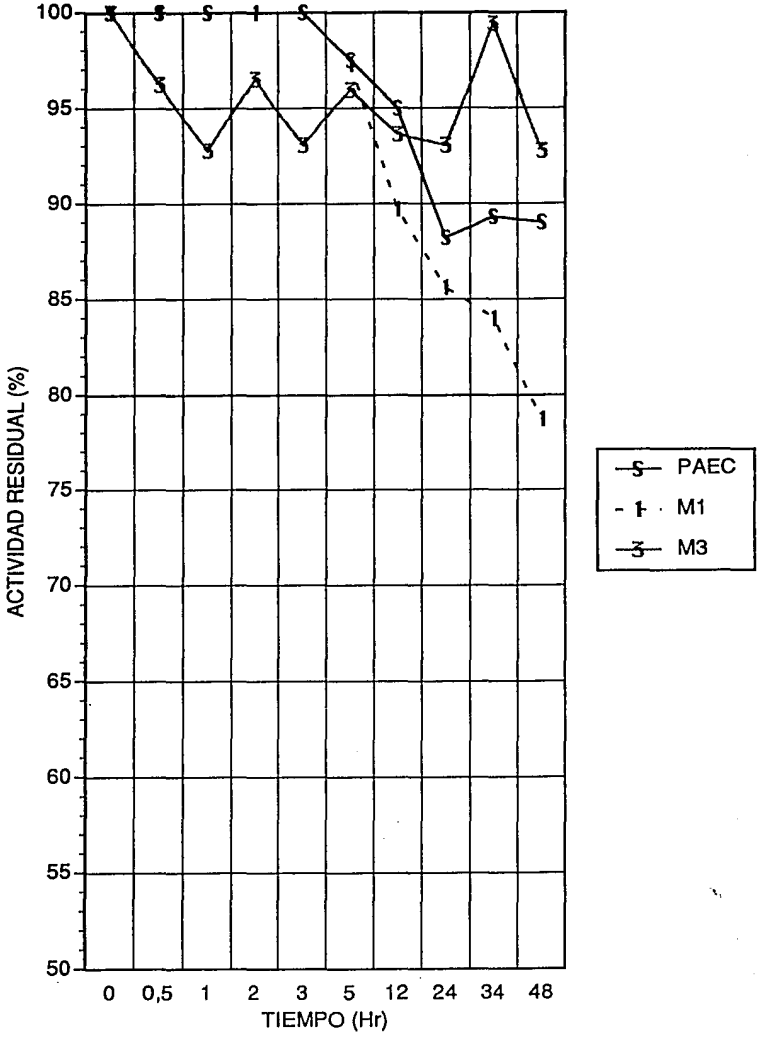
pH
PAEC
M1
M3

3A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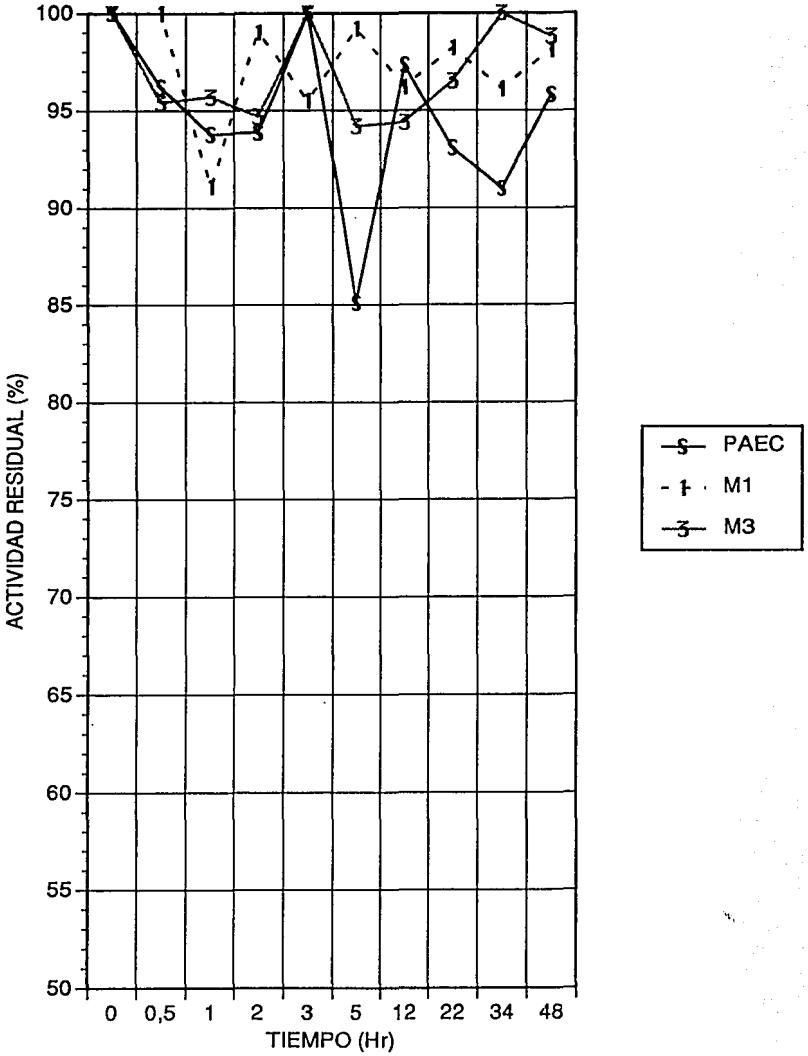
TIEMPO (Hr)
PAEC
M1
M3

3B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ACTIVIDAD RESIDUAL (%)
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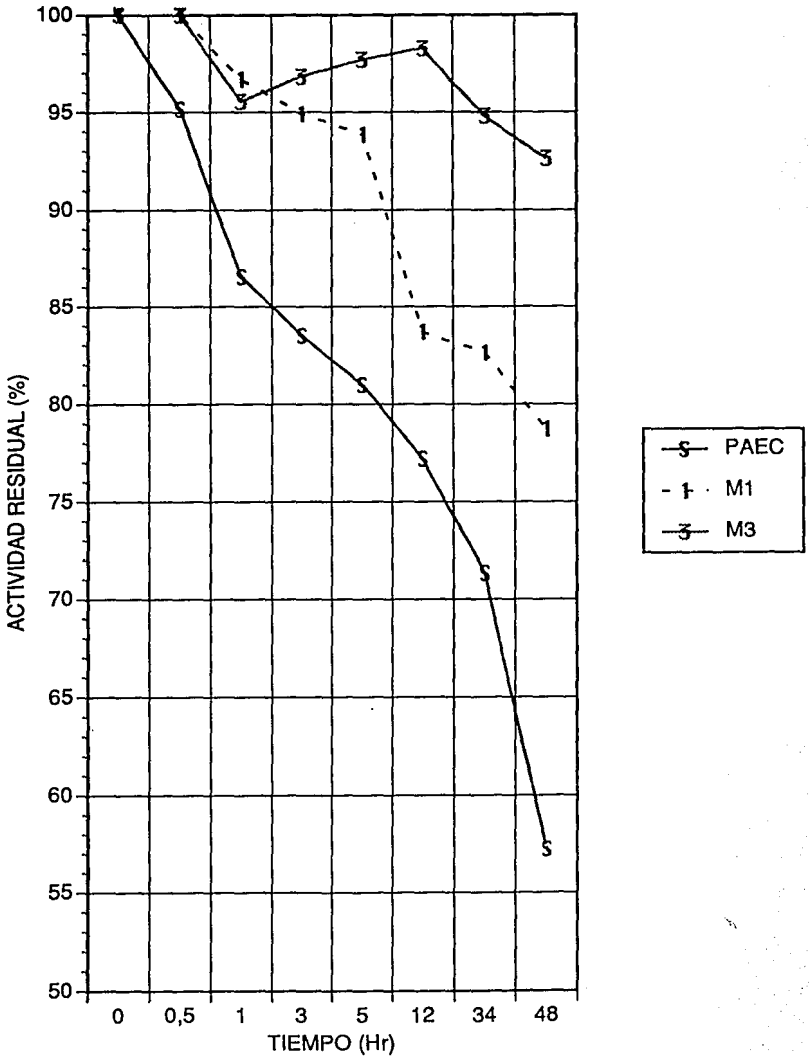
PAEC
M1
M3

3C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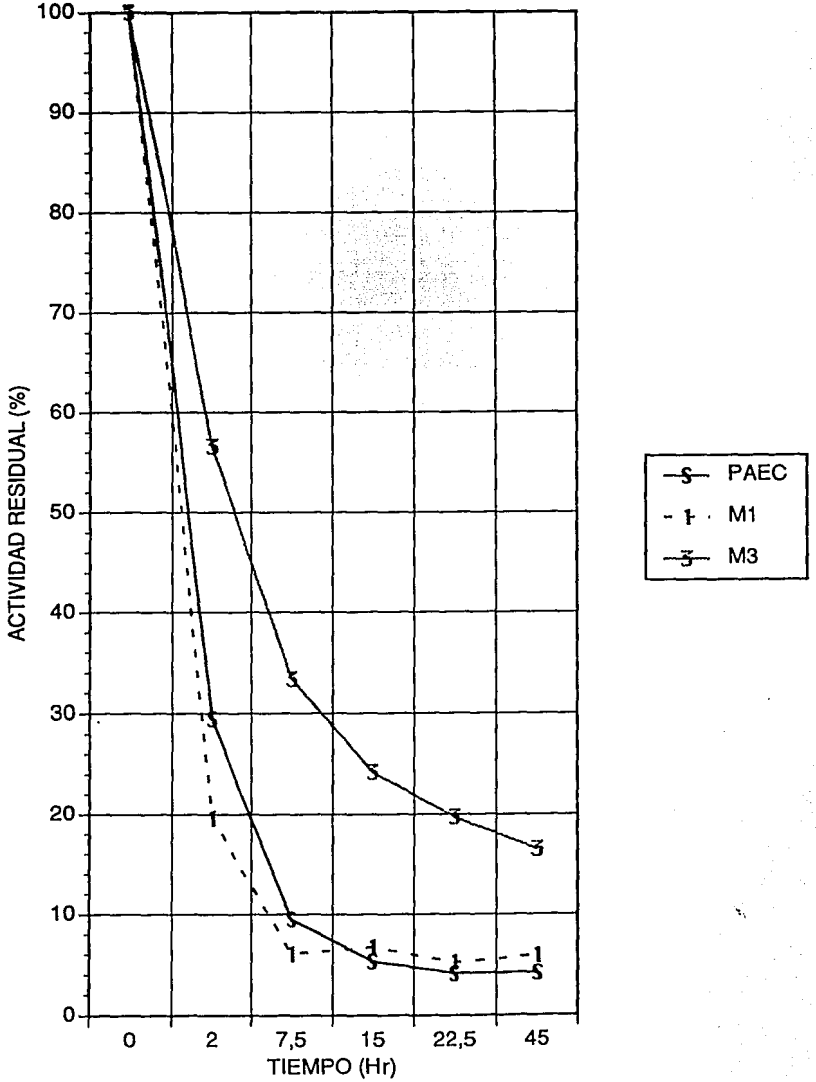
PAEC
M1
M3

3D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ACTIVIDAD RESIDUAL (%)
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22,5
45
TIEMPO (Hr)
PAEC
M1
M3

TRABAJOS SOMETIDOS A PUBLICACION

Combinatorial libraries of proteins: on the efficiency of mutagenesis techniques.

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Abstract

A number of considerations are made on the efficiency of mutagenesis techniques used in protein engineering, particularly those that include a random component. The expected outcome of different protocols is analyzed using computer programs. Special emphasis is made on the effect that the degeneracy of the genetic code has on the bias of the representation of amino acid replacements. The consequences of using alternative methods is analyzed in terms of the likelihood of obtaining under-represented amino acid substitutions in mutant libraries. A consideration is also made of the outcome of combinatorial mutagenesis experiments with regard to the size of the amino acid window that could be sampled with different methods.

Keywords: polymerase chain reaction; phage display; protein engineering; combinatorial mutagenesis; *in vitro* evolution.

Introduction

Since the advent of recombinant DNA technology the study of protein structure and function has received tremendous impulse. The capacity to implement any desired amino acid (a.a.) replacement on proteins has taken the study of their sequence, structure and function relationship from what was essentially an observation stage into the realm of experimental science.

Variant proteins have been obtained by geneticists for a long time, with interesting phenotypes being selected out of very large numbers of cells. The lesions, at the DNA level, could then be identified after significant work. Subsequent analysis of the corresponding differences at the polypeptide level provides the bridge to the functional consequences of the mutation. Recombinant DNA permitted the sequence analysis of many mutants obtained in this way, but also took us to a new scenario, in which the production of the mutations is subject to control by the experimenter.

The use of site-directed mutagenesis techniques is widespread and has now became routine to approach the study of protein structure and function by protein engineering. Moreover, the power of recombinant DNA based mutagensis has driven research to ever more ambitious schemes for the introduction of a great deal of variability, specially since the development of powerful screening systems (33,38)).

Several approaches have been utilized which incorporate such random component. Indeed, in the absence of a sufficienly robust methodology to predict the precise outcome that the alteration of one or more residues of a protein may have on its structure and function, these approaches are likely to be employed for quite some time. The use of combinatorial libraries in present day biotechnology seems an important way to develop novel molecules (11,12). Indeed, in recent years, mutagenesis approaches based on a random component, coupled with the power of recombinant DNA methods and a suitable assay, have yielded very fruitful insights in the absence of precise hypotheses regarding the outcome of the experiments. Cassette mutagenesis (32), regional mutagenesis (4,20) "spiked oligonucleotides (oligos)" (19) and combinatorial mutagenesis (29,31,32) are terms referring to such approaches.

In cassette mutagenesis, a target region is selected and suitable adjacent restriction sites are

found or introduced. A synthetic DNA duplex is made, containing the desired mutation(s) and subsequently cloned, replacing the wild type sequence. Regional mutagenesis is achieved by several methods. In general the protein of interest is subjected to any of several treatments (chemical or enzymatic), using conditions that introduce a controlled rate of alterations per nucleotide. The product of such reactions is then cloned. The term "spiked oligos" has been used to refer to methods employing synthetic oligos in which some amount of a mixture of different bases is included at predetermined positions, "spiking" the wild type base during the synthesis. The oligos are then used to prime an extension reaction with it's final incorporation into heteroduplex DNA molecules, which are cloned. The goal of combinatorial mutagenesis is to generate mutants with several a.a. replacements per protein. A region or "window" is defined, and the rate of mutagenesis is set as high as necessary for the multiplicity of replacements desired. This has generally been done using cassettes.

Since the usual mutagenesis unit is the nucleotide and the protein-variation unit is the a.a., the genetic code, which relates the two, poses specific constraints on the process of creating collections of protein variants (37) . This facet of mutagenesis is only superficially mentioned in much of the literature and only a few papers have dealt with it specifically (19,31). These considerations become particularly apparent when one considers the astronomically high numbers of variants that can be easily generated by recombinant DNA methodology. A combinatorial mutagenesis scheme involving a mere 7 a.a. residues would generate over 10^9 variant proteins under ideal conditions, which is above the practical limits of currenly available screening methodologies (3,22,33). Given that the number of residues directly involved in molecular recongnition and/or the catalytic function of any given protein is likely to be higher than 7, there is a need for optimizing mutagenesis procedures in order to perform the screening on the best possible collection of variants. Indeed, some recent literature explores various ways to achieve that goal (6,8,15,21,24,35).
In this paper, we analyze the expected outcome of diferent mutagenesis schemes, as reflected in the frequency, distribution and variety of a.a. replacements. We focus primarily on the efects of various ways of "doping" oligonucleotides to make degenerate mixtures and also on the variables involved in schemes that aim at non-saturating conditions. Such conditions have

been utilized and analyzed before from other perspectives (9,17,19) and constitute an atractive way to better sample the sequence space, with a collection more likely to contain interesting variants (8,37). The study we present here aims to provide a general reference and some procedures to evaluate and compare mutagenesis regimes in terms of the expected outcome at the level of amino acid replacements, rather than at the DNA level.

Results and Discussion

Computer programs were written for calculating the expected outcome of mutagenesis schemes. In relating our calculations to the actual experimental situations, it should be kept in mind that several assumptions/simplifications were made: for a chemical or enzymatic mutagenesis procedure a random distribution of base changes was used; that is, replacement of any base by AGC or T had equal probability; similarly, for a method utilizing oligos as mutagenic agents, the probability of a base being incorporated into the oligo was considered to be related only to its concentration on the coupling mixture during the synthesis cycle.

We first considered the average frequency of a.a. replacements expected when each of the 61 amino acid-coding base triplets were mutated, at a given rate, at any of the three codon positions. This can be called NNN mutagenesis and it is applicable to the idealized form of any method not employing oligos as mutagens (chemical or enzymatic, for instance). It would also correspond to a spiked oligos approach in which the DNA synthesis is performed using mixtures of the wild type base in one vial, and equimolar amounts of the four bases in another vial (19).

As plotted in Fig. 1A, the frequency of mutant protein vs mutant DNA rises in a nonlinear fashion, and varies depending on the method used. The plots show the values as an average of the 61 sense codons grouped according to the amino acid they code for. In comparing the properties of different methods, we used an approach aimed at simplifying the evaluation of relevant parameters involved in current mutagenesis designs (see Discussion section). This approach considered the actual rate of a.a. replacements, rather than the rate of nucleotide replacement. This parameter is therefore used in several of the calculations that follow. Another important consideration, the bias of the frequency expected for each of the 20 possible a.a.s has been discussed (37), and was analyzed here in a simplified fashion. We defined a "richness"=R for each of the mutagenesis conditions. R has a value of one in the ideal case when, for every codon, mutagenesis is done such as to obtain a frequency of 1/20 for each a.a. in the mutant fraction (such "ideal" case could be attained by using triplets as

mutagenic units, see below). Otherwise, the value of R decreases and reflects an imbalance or bias in the distribution of a.a. replacements. To illustrate this, in Fig. 2 we show the expected distribution of a.a. replacements when an arginine codon (AGG) is analyzed. It is clear that, at a low mutagenesis rate, the representation of different a.a.s is very strongly biassed (low values of R) and at a higher rate the imbalance is much less pronounced (higher values of R). Fig. 1B depicts the significant change in the "richness" values of the distribution of a.a. replacements as the rate of mutagenesis increases. This obviously reflects the fact that, for a given codon, some a.a. replacements can arise with a single base pair change whereas others require double or triple base pair changes. Even at extreme conditions, the attainable richness stays lower than one since some codons are still over-represented at a ratio of 3/1.

We next compared the expected outcome of experiments utilizing all four bases at each of the three codon positions (NNN) with those using only G or C at the third, "wobble", base (NNG/C). Such a scheme has been utilized (11,32) as a means to reduce the appearance of termination codons (from 3/64 to 1/32) and to make the distribution of some a.a.s more even (met and trp from 1/64 to 1/32); the approach requires synthetic DNA. A further variation should be considered here, namely that the experimenter could chose to introduce some proportion of the wild type base at the third position of the codon or, alternatively, a 100% contamination of the G plus C mixture could be used (both methods would afford a sampling of all a.a. replacements); only results of the latter approach are given, since results from the former are virtually identical to the NNN alternative.

The results (Fig. 1B) show that better richness values are afforded by the NNG/C method, specially at equivalent, low a.a. replacement rates. It should be noted, however, that codon usage would be significantly changed with this approach, as every mutant codon would end with G or C, regardless of the original codon present. The difference in richness values translates into notable differences when these mutagenesis approaches are performed looking for multiple a.a. replacements (see below). For instance, a mutagenesis rate of 0.2 will produce an a.a. replacement rate of 0.1 for both approaches (see fig. 1A), but with a clearly different richness value (NNN= 0.1, NNG/C= 0.3). On the other hand, at saturating conditions, the two methods are very similar.

We then looked at the expected difference of mutating idealized genes with each of the

following compositions: a) a non-biased codon distribution; b) a codon distribution corresponding to highly expressed *E. coli* genes; c) genes composed by selecting codons, one for each a.a., having the highest richness in the expected distribution of a.a. replacements. Mutagenesis in such fashions could be attained with completely synthetic genes where the codons are controlled at will. We found that the differences are not very significant (data not shown). It would be expected, however, that in mutagenesis experiments targeted to small regions, the choice of initial codons may indeed have an impact on the results.

It has been argued (37) that using triplets as mutagenic units would result in significantly better mutant collections. Such an approach would afford a richness of 1 (by employing, at the DNA synthesis stage, an equimolar mixture of 20 codons, one for each a.a., in the mutagenic vial), irrespective of the mutagenesis rate. A glimpse to fig. 1 shows that the use of trinucleotides would be most advantageous at low mutagenesis rates and this advantage is again broadened when one is looking for multiple a.a. replacements. A similar, constantly high richness value could be achieved with NNN or NNG/C approaches, but employing a more elaborate protocol (6,15,21), see discussion section).

Mutagenesis of large DNA segments, such as a regional mutagenesis scheme (4) over the whole gene, or a method utilizing long "spiked oligos" (23), are typically implemented utilizing relatively low mutagenesis rates, targeted at obtaining populations of genes with low proportions of multiple a.a. replacements. The question could be formulated in terms of the probability of finding a particular type of mutant given the fraction it represents in a mixture and the size of the population accesible for screening. With this in mind, we decided to analyze the population sizes required for the sampling of single, double and triple a.a. replacements, as a function of the length of the target region (window size, see methods section), with the various mutagenesis methods. The results are expressed as the reachable window size for each method. We set a cutoff value at 95% probability of finding an under-represented mutant when looking at 10^2-10^8 clones (the lower limit relates to the use of an elaborate assay, and the upper limit to the transformation efficiency attainable by electroporation in *E. coli*). The results, shown in Fig. 3, demonstrate that there are noticeable differences among the three approaches in certain conditions. For larger window sizes and higher multiplicities, the differences become more pronounced. It is worth noticing that only

when a high density screening is available (allowing to look at, say, 10^7 clones by selection) is it possible to sample single replacements in gene-sized windows with any method. Further, it could be feasible to sample all possible triple replacements within a ten-residue window by using trinucleotide mutagenesis and selection (>10^7 clones).

When the researcher focuses on small mutagenesis windows, such as residues near the active site of an enzyme (13), a hydrophobic core(25) or the relevant surface of a protein ligand (7), for instance, additional considerations apply. In these cases, different distributions of a.a.s may be obtained by selecting the composition of the base mixture used at each codon position (1,8,26). Such "intelligent" mixtures could also be implemented using a trinucleotide approach, with potentially better results.

Conclusion and Recommendations

Based on this study, we can draw some conclusions and provide some general recommendations for mutagenesis experiments.

The analysis of the a.a. rather than nucleotide replacement rate allow us to represent useful experimental parameters (window size, mutagenesis rate and library size), in order to improve designs of mutagenesis experiments. For instance, taking into account the differences between the three mutagenesis approaches analyzed here, one could decide the best mutagenesis rate parameter; also, this analysis could be helpful to evaluate, with a low accessible library size, the probability of success in looking for desired mutants. These and other experimental strategies could be analyzed just varying the parameters included in our calculations. A computer program intended to support that kind of analysis is available from the authors.

In a regional mutagenesis approach, perhaps the main difference between the chemical, enzymatic and the "spiked oligos" methods, is related to the degree of control over the rate of mutagenesis and the degree of true randomization of each base in the mutagenesis window. For both parameters, the results should be better with the latter method. These three methods, as they stand today, suffer from the drawback imposed by the genetic code, which, in practice, limits the representation of a.a. replacements to a subset (eg. about a third of all possibilities in small libraries aimed at one or a few replacements per gene;(37)).

Depending on various considerations, different mutagenesis schemes could be used for different questions and systems. Clearly, in some cases, sufficient knowledge is available to draw meaningful information from site-directed and site saturation mutagenesis experiments (18,28,41,42); in such cases, the methods are well established and do not represent a serious limitation.

On the other hand, although we know that very significant changes on the properties of enzymes can be afforded by a few a.a. replacements (10,17,34), we are still incapable of predicting the outcome of most of such variations. It is also clear that, in many instances, more than one replacement will be necessary before an observable (desirable) change appears (10,14,18). Very interesting mutants have been found through the judicious choice of "windows" of residues, which are then mutated, either in an incremental, site-directed manner (34) or using combinatorial, cassette mutagenesis approaches (25). In this realm, methodological bottlenecks begin to appear; as the targeted mutagenesis window grows larger, the issue of efficiency becomes more pressing. One would ideally want to preserve the richness of high rate mutagenesis, while keeping multiple replacements under control (37). For instance, sampling single and double a.a. replacements within a window of, say, 20 residue positions, would be highly desirable. Using the first derivative of the binomial distribution equation (27), one finds that the optimum a.a. replacement rate for this condition is in the order of 0.1 which, in turn, corresponds to low richness values with conventional methods (see Fig. 1B). Similar considerations apply all the way up, until the mutagenesis "window" is the DNA coding for a whole protein or protein domain. This situation could be further illustrated by considering the reachable window size when sampling difficult mutants (Fig. 3).

One obvious way to improve this situation would be to develop methods for "spiking" oligos at the codon level(37,43): some steps in this direction have been taken(31,35). We are currently working on synthetic DNA approaches that accommodate this, while maintaining compatibility with existing chemistries and automated synthesis machines. Another way to achieve a similar result consists in performing the oligo synthesis with removal and remixing of the solid support: a fraction of the resin is taken out, reacted for three cycles to achieve a

100% NNN or NNG/C regime, while the remainder is separately reacted with bases comprising the wild type codon; the two fractions are remixed and the procedure repeated for as many codons as necesary. We, as well as others (6,15,21), have successfully used this approach. Unfortunately, the method is too cumbersome for large mutagenesis windows.

In addition to the previous improvements, a desirable goal would be the combinatorial mutagenesis of windows of a.a. residues which are close in three dimensional space, but not contiguous in the sequence (e.g. residues clustered around the active site, a hydrophobic cluster, a protein-protein interface). Recent advances in mutagenesis methods, especially those based on the Polymerase Chain Reaction (PCR) have made such a goal feasible (16,29,30,40). As we stand today, it is not possible to formulate precise predictions about the structural and functional consequences of residue substitutions in proteins (14). Rather, it seems that a combination of crude predictions and hindsight could be applied, together with an empirical approach to derive new or improved functions from existing proteins. Recent developments in the derivation of rough structural models of proteins, such as fold recognition algorithms (2,36) are very encouraging. Another exciting development is the utilization of the bacteriophage display of mutant protein libraries (38), which allows the screening of vast numbers of protein variants for specific binding attributes. Clearly, the most efficient mutagenesis methods will constitute an important component of the battery of methods required for the artificial evolution of proteins.

Methods.

All computer programs were written in Turbo Pascal 5.5 (Borland-Osborne) and run on an IBM PC compatible machine or in C ANSI, and run on a DECstation 3100 (Digital Equipment Corp.).

The programs considered each of the 61 sense codons that comprise structural genes. The outcome of a mutagenesis experiment was calculated based on the probability of finding a base, different from wild type, on each of the three codon positions. Such probability depends on the mutagenesis method employed. The resulting distribution of DNA sequences was then evaluated in terms of the distribution of a.a.s it coded for.

Amino acid replacement rate.

The quantification of this parameter was done in therms of the expected frecuency of each codon in the mutant populations generated. This was assumed to be proportional to the nucleotide concentration on the mutagenic mixture. Therefore, for each base at any codon position:

$F_b=F_{bW}+([1-F_W]*0.25)$ (1)

where:

$F_{b=}$ Nucleotide concentration for a specific base at eahc codon possition; $F_w=$ Wild-type codon rate in mutagenic mixtures; $1-F_w=$ Mutagenesis rate; $F_{bw}=$ Nucleotide concentration for the base corresponding to the wild codon on the mutagenic mixture.

This formula was used for the so called NNN mutagenesis scheme at all three possitions. For the NNG/C mutagenesis case, the frequencies for the first two bases of a codon were calculated with equation (1) and a frequency of 0.5 for each of the bases C and G was used at the third position (see the Results section for considerations on the experimental conditions pertaining to these calculations).

A set of calculations were performed for several mutagenesis rates on the two mentioned

approaches. For each codon, 64 or 32 frequency values were generated, corresponding to each possible triplet. The values of synonymous triplets were then added together, giving a total of 20 frequencies (A_M), one for each a.a. replacement (including stops), and one frequency (A_W) for the wild type a.a..

Fractions $1-A_W$, over all 61 codons, were averaged for each mutagenesis rate, giving the expected a.a. replacement rate.

In the case of a highly expressed gene, weighted averages were taken for the codons, using the values given by Wada et al. (39). An "optimal" gene was also considered, including only the codon with the maximum richness (see below) for each a.a..

Richness value.

To each codon and mutagenesis condition, we also associated a "richness" value:

$R= \Sigma\, r_M/20$ (2)

where

$r_M=[(1-A_W)/20]/A_M$ and i represents the (3)

or codon.[poner subíndices]

$r_M=A_M/[(1-A_W)/20]$ (4)

Equation (3) was used for over-represented a.a.s (with frequencies of more than 1/20 of the total a.a. replacement rate) and equation (4) for under-represented a.a.s. Thus, $r_M \leq 1$. An average richness was then calculated for each contidion over the 61 sense codons.

Reachable window size.

We employed the equation (5):

$N= [\ln(1-P)]/[\ln(1-S)]$ (5)

to relate the population size, N, with the probability, P, of sampling a mutant represented at a fraction S in the population. P was set to 0.95 and values of S were derived for $N=10^2$ to 10^8. Hence:

$S= 1 - e^{(\ln(1-P))/N)}$ (6)

The values for S were then used to calculate a reachable window size, n, using equation:

$S=A_W^{(n-m)}(1-A_W)^m(A_M/[1-A_W])^m=A_W^{(n-m)}A_M^m$ (7)

where

m= number of a.a. replacements per window.

Corresponding values for $A_M=1-A_W$ were calculated by interpolation, from a polynomial expression fitted to the data of A_M *vs* $1-F_W$ derived in previously.

Hence:

$n= [(Ln(S) - m*(Ln(1 - A_w)))/(Ln(A_w))] + m$ (8)

Values for A_M were those of a.a. replacements requiring two base pair changes, as per eq. (1): $A_M=[F_{bW}+(1-F_W)*0.25][(1-F_W)*0.25]^2$ for NNN mutagenesis and $A_M=0.5*[(1-F_W)*0.25]^2$ for NNG/C mutagenesis. We have analyzed only the case of two base pair changes in order to directly compare the NNG/C and the NNN approaches; in the NNG/C approach the third codon possition is saturated with G/C and therefore it is meaningless to consider triple base pair changes.

Program for analizing diverse mutagenesis conditions

We developed a user interface for the application of the equations described above. The user specifies a set of conditions and the program calculates the unspecified variable or variables. One can ask, for example, the following question: what size window can I sample when looking for triple replacements at 85% probability if I am able to screen 10^5 variants: compare the sizes for the NNN or the NNG/C methods. The program was developed in Visual Basic (Microsoft Corp) for the PC Windows environment and it is available from the authors.

Acknowledgements

We are thankful to Sonia Caro for secretarial assistance and to Nina Pastor for critically reading the manuscript.

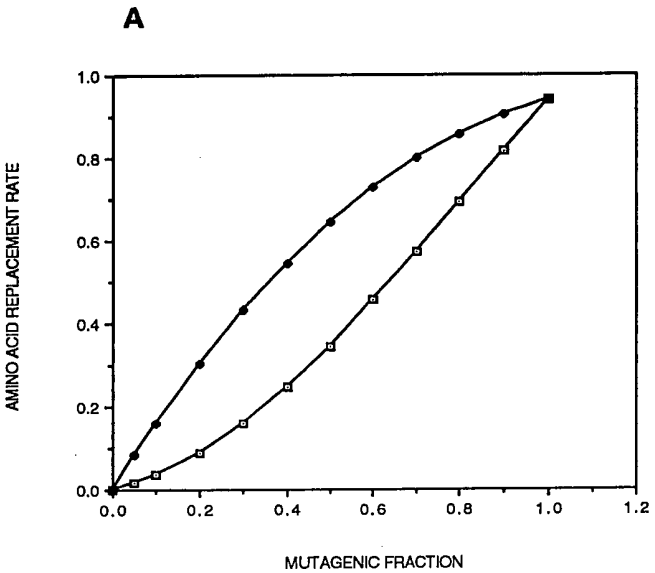
This work was supported, in part, by Grants from PAPIID, DGAPA/UNAM and from PEMEX.

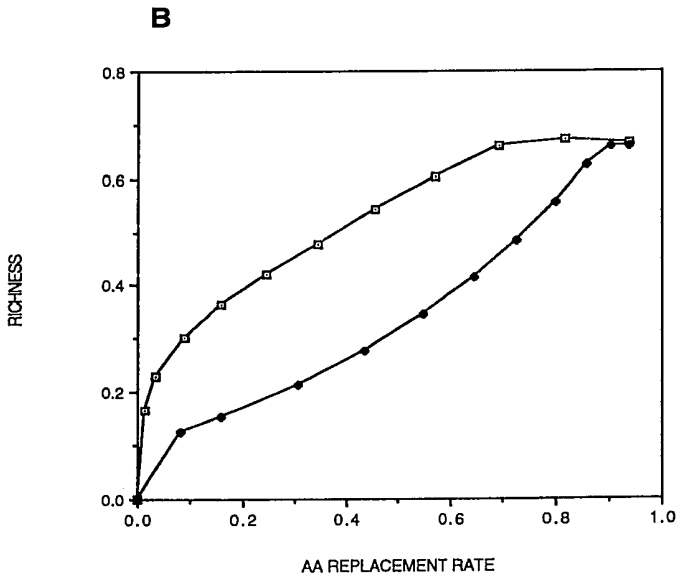
Figure legends

Figure 1. Comparisson of the amino acid replacement libraries produced by NNN and NNGC mutagenic approaches. The variables mutagenic fraction, amino acid replacement rate and richness are defined in the Materials and Methods section.

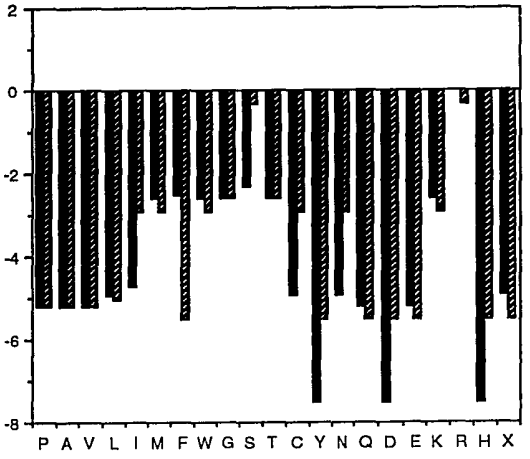
Figure 2. An example of the expected distribution of amino acid replacements at different rates of mutagenic fraction. Values for the AGG arginine codon are shown for both mutagenic approaches: NNN and NNGC, for A), a mutagenic fraction of 0.1 and B), a mutagenic fraction of 0.5.

Figure 3. Comparison of the reachable window size, as a function of accesible library size and amino acid replacement rate, of diverse mutagenic approaches. NNN (light gray lines), NNGC (heavy gray lines) and trinucleotide (black lines) are shown. Values for single, double and triple amino acid replacements are shown in panels A, B and C, respectively.


A
AMINO ACID REPLACEMENT RATE
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
MUTAGENIC FRACTION


B
RICHNESS
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
AA REPLACEMENT RATE

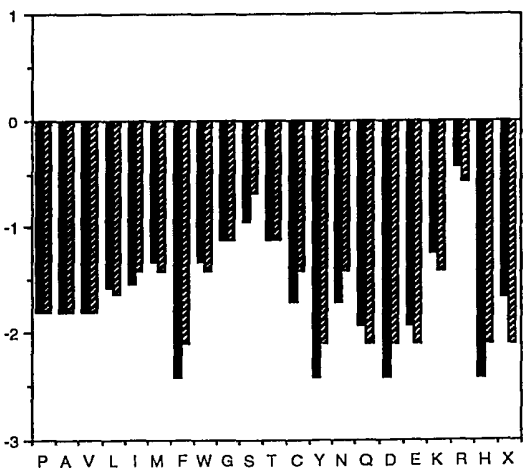
A



LOG (CALCULATED FREQUENCY)

AMINO ACID

B



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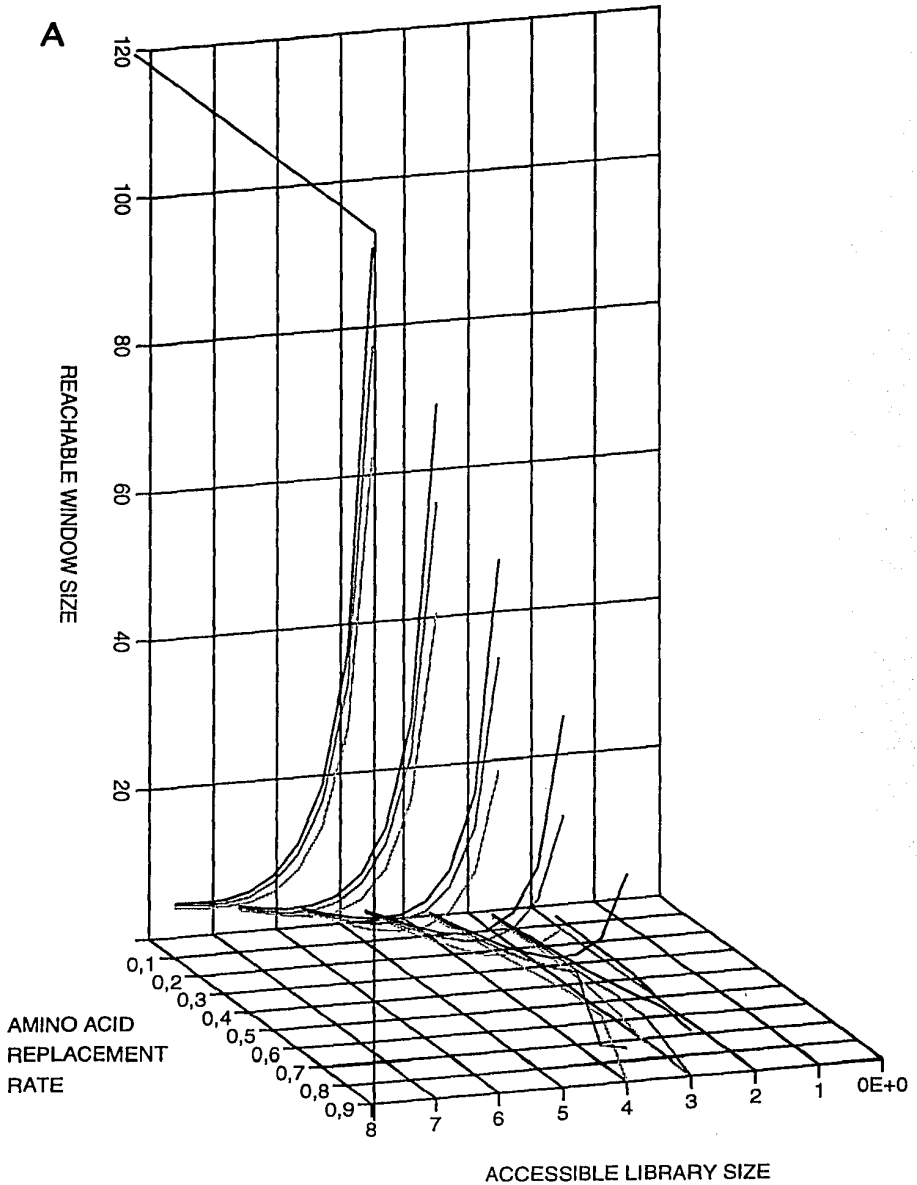
AMINO ACID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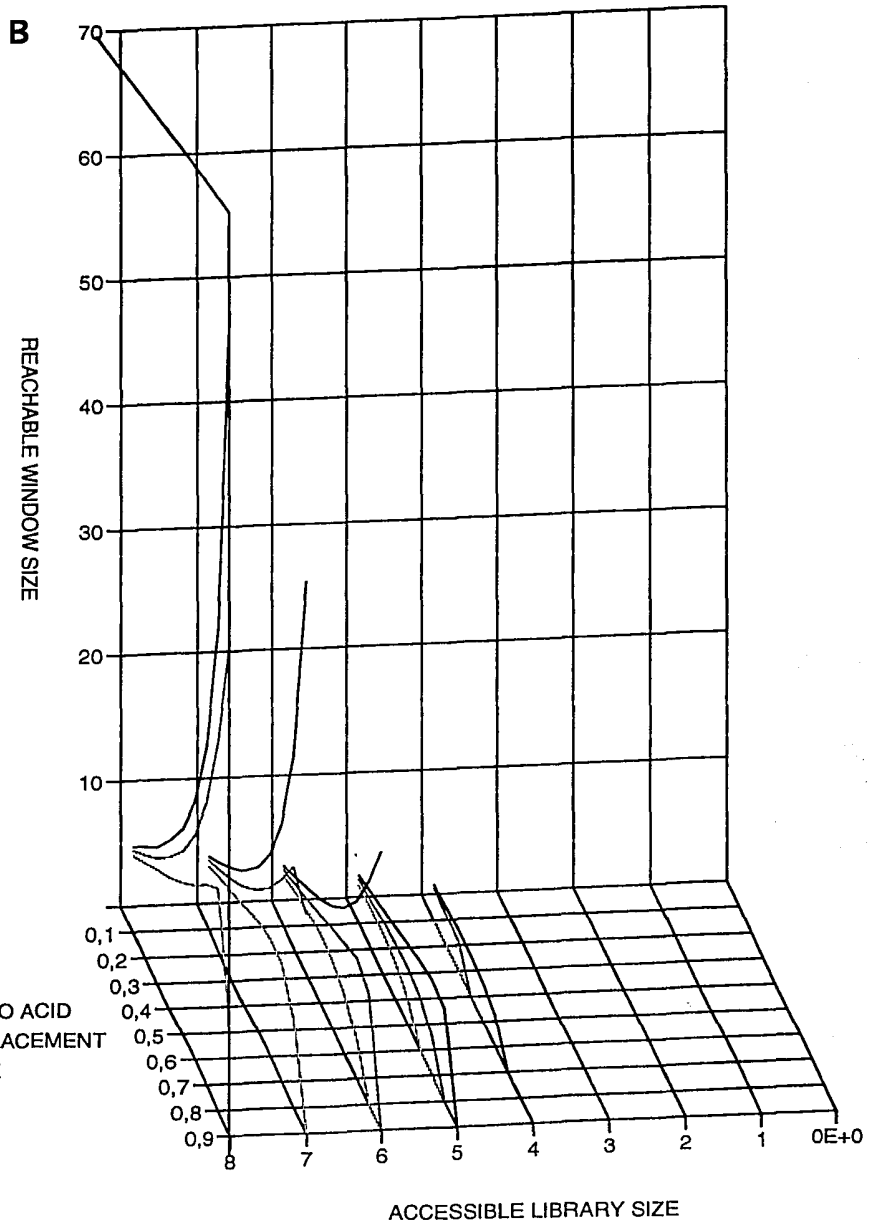
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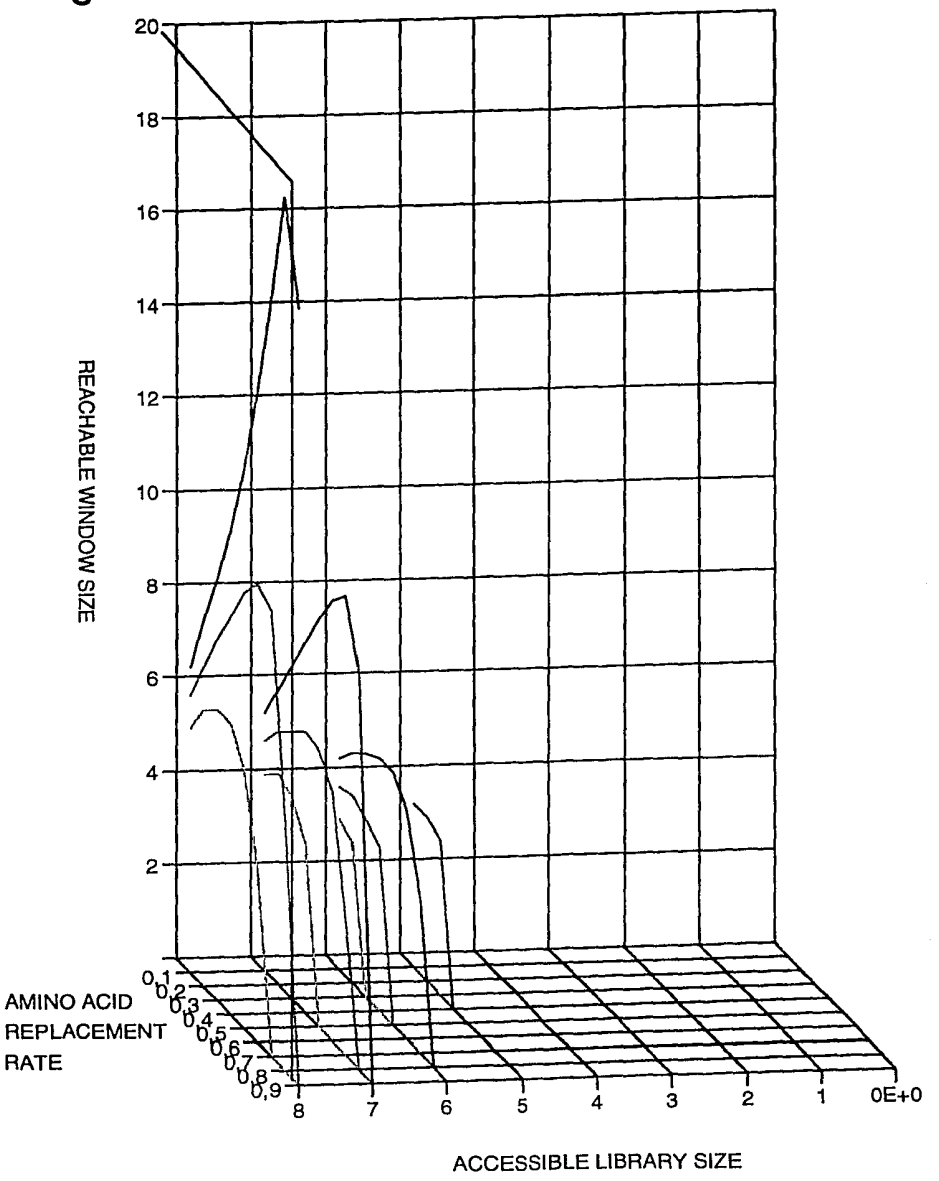
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Mutant *E. coli* penicillin acylase with enhanced stability at alkaline pH.

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Running title: Stability mutant of penicillin acylase.

ABSTRACT

The increased stability at alkaline pH should be a valuable attribute for the utilization of penicillin acylase in bioreactors employed to convert penicillins into 6-amino-penicillanic acid, a precursor of semi-synthetic penicillins. In these systems, base is added for pH control, which results in local alkaline conditions that promote enzyme inactivation. In this work, the gene coding for *E. coli* penicillin acylase was subjected to oligonucleotide-directed, random mutagenesis at regions coding for amino acids predicted to be at the surface of the enzime. The resulting mutant library, cloned in *E. coli*, was screened by a filter assay of the colonies for the presence of penicillin acylase activity with enhanced stability at alkaline pH. Characterization of selected clones revealed the presence of a mutation altering the surface charge of the protein. *In vitro* experiments demonstrated a near two-fold increase in the half life for the enzyme activity at pH 8, as compared to the wild-type with a comparable specific activity at several pH values.

Keywords: protein stability, protein surface charge, 6-amino-penicillanic acid, biocatalysis.

INTRODUCTION

Penicillin acylase (EC. 3.5.1.11) is one of the few immobilized enzymes in use at industrial scale. Its main application is in the annual production of more than 4,500 ton of 6-amino penicillanic acid (6-APA), one of the key raw materials for the production of semisynthetic penicillins. The annual sales of penicillin acylase biocatalysts is around 6 million dollars, only below those of glucose isomerase, and the market must grow to satisfy a demand of 7,000 ton of 6-APA in the year 2,000 (25). More than 15 companies all over the world have developed immobilized biocatalysts for this application, most of them using the purified enzyme from *E. coli* (24).

The enzyme exhibits interesting specificity and catalytic properties and it is therefore potentially useful in other applications in biocatalysis. Besides the cleavage of the acyl side chain of penicillin, it also catalyzes, at a lower pH, the N acylation of 6-APA with analogues of phenyl acetic acid (PAA) to yield the corresponding semisynthetic antibiotics such as ampicillin, amoxycillin, oxacillin, etc.. This catalytic property has been recently examined in the presence of cosolvents in search for a biocatalytic alternative to the actual chemical synthetic processes (2,7,8). The enzyme also presents stereospecificity towards a number of nucleophiles and may be useful in the resolution of phenylacetyl derivatives of compounds of industrial interest such as aspartame, insulin and a wide variety of aminoacid and peptides esters (11). Finally, the use of penicillin for the protection/deprotection of amine or hydroxyl groups in

sugars and peptides has also been reported (10).

There are many reports in the literature concerning penicillin acylase biocatalysts, including the production (or over production) of sufficient quantities of the enzyme, its kinetic and physicochemical characterization and the development of stable derivatives for its application in bioreactors. Penicillin acylase has been over produced from mutants obtained by chemical mutagenesis and using genetic engineering. In particular a 2-20 fold increase in PA production has been obtained for the *E. coli* enzyme (16,17). The combined strategy of a genetically modified strain with specific culturing techniques such as high cell density continuous culture (14) or fed batch at constant specific growth rate (O.T. Ramírez, personal comunication), has resulted in an improved productivity. However, the main drawback of penicillin acylase is its long-term stability during operation: stable derivatives have been produced in EupergitC, Sephadex G-200, Polyacrylonitrile fibers, cellulose (25) etc. with theoretical productivities up to 1000 Kg of 6APA produced per Kg of biocatalyst, in more than 500 cycles of enzyme reuse. Reactor type and industrial reaction conditions are selected mainly based on long term enzyme stability: the biocatalyst is never used under conditions for maximum reaction rate in commercial processes, and a compromise is made to consider unstability factors, including those produced by substrate and products inhibition. Among these factors pH is particularly important as the acidification resulting from the production of PAA and the oscilations caused by the neutralization system affect not only the rate of the forward reaction but also the long term stability of the biocatalyst.

For instance, the half life at 37°C of a penicillin acylase immobilized in an epoxyacrylic resin, stored at pH 7.5 is 2880 h but it falls to 869 h at pH 6 and to 321 h at pH 8.5 (21). When the half life is determined during batch operating conditions with pH regulation at 7.5 ± 0.1, the half life decreases to 1155 h. The lack of stability towards pH is also a problem in the potential industrial application of PA for the synthesis of modified penicillins. Due to these properties and to its current and potential industrial applications, penicillin acylase is both an interesting and an important model to develop structure-function relationships in order to design protein engineered enzymes with improved characteristics.

The use of protein engineering for the alteration of protein stability has been successful in many cases (5,12,19,28,29,31). Ideally, a three-dimensional structure of the protein provides a starting point for the design of alterations that can potentially bring about a change in the desired property. Unfortunately, a crystallographic structure of penicillin acylase is not available yet. One has then to rely on predictive methods and general theoretical considerations to guide an experimental mutagenesis protocol. Also, in the absence of precise hypotheses regarding specific residues and alterations, a simple screening permits the scoring of many different mutants, generated using protocols with a random component (3,4).

The objective of this research was to create a collection of mutant penicillin acylases, potentially enriched for alterations influencing their stability at different pHs. For this purpouse we took into account the generally accepted notion that pH stability of

proteins is related to their surface charge and that it could be expected that a protein is most stable at it's isoelectric pH (1,12,23). We used secondary structure and surface propensity (hydrophilicity) predictions to define regions likely to be at the surface of the protein. We then designed a mutagenesis protocol aimed at creating alterations of amino acid residues at such regions, such as to modify their charge. The resulting mutant collections were then screened utilizing a simple, filter paper assay, where the product of the enzyme reaction generates a colored product (4).

MATERIALS AND METHODS

Design of oligonucleotides.

The mutagenic oligonucleotides were synthesized in the Molecular Synthesis Unit of the Institute of Biotechnology/UNAM. The sequences of the oligos were designed so as to include variability, biased towards positively charged residues, at two or three codons located at each of the selected regions in the PAC gene (oligo design will be published elsewhere).

Mutagenesis protocol.

The mutagenesis was designed to introduce mutations in two zones of the *pac* gene. This mutations correspond to surface residues in the protein as predicted by the Kyte & Doolittle algorithm (13) (see Results and Discussion section): one in the alpha subunit and other in the beta subunit (oligo 1 and 2, respectively). Oligos 3, 4 are based on sequences adjacent to the M13mp and pUC series of vectors: they are actually extensions of "universal" and "reverse" M13 sequencing primers, designed to have similar melting temperatures. They are able to function with any DNA cloned on such vectors (18) . Oligo 5 permits selective amplification of mutant PCR products (see Fig. 1).

PCR mutagenesis was then performed as described (18) with the following conditions: 20 cycle of 1.5 min al 92 C; 1.5 min at 55 C and 3 min at 70° C, followed by a final reaction of 15 min at 70° C. PCR reactions where mutagenic oligos were employed

included an initial cycle with an annealing temperature of 50° C.

Stability screening protocol.

Bacteria cell from *E.coli* JM101 were transformed by the plasmids containing the wild-type and mutant *pac* genes (plasmids pBGS19pac (21) and pBGSM1, respectively, see Fig. 1) by electroporation into *E. coli* JM10, and grown in plates at 37 C in LB broth with kanamycin at 25 mg/ml.

The colonies were transfered to Whatman 540 filter paper in triplicate, and subsequently lysed with chloroform vapors for 30 min (this attaches cellular and periplasmic proteins to the filter). Each filter was submerged in a different buffer solution: 0.1M citrate buffer, pH 5.0, 0.1M phosphate buffer, pH 7.8 and 0.1M glycine buffer, pH 10, during 1 or 3 hrs.

The filters were rinsed 3 times with 2% penicillin G solution for 30 min at 37° C and then covered with the PDAB solution. PAC activity intensifies the yellow color of around the colony producing a dark yellow coloration on a pale yellow background.

Cloning and expression of mutant proteins.

The mutant fragments obtained from PCR were cloned into pBGS19PAC: the beta subunit was introduced as a *Bgl*II-*Eco*RI fragment and the alpha subunit fragment as a *Hind*III-*Bgl*I fragment (see Fig. 1).

DNA sequencing

The mutant pac gene was sequenced using Sequenase (USB) following the vendors indications.

Enzyme activity

Enzyme activity was measured with 2% penicillin G ((Orfaquim, S.A.) in phosphate buffer 0.1 M, pH 7.8 at 37°C. The initial production rate of 6-APA was followed by the p-dimethylaminobenzaldehyde (PDAB) method (21). One activity unit (U) is defined as the amount of enzyme producing 1 mmol of 6-APA per min.

Enzyme concentration

Active-site titration of PA enzyme concentration was determined by direct using phenylmethanesulphonyl fluoride (PMSF). The enzyme was incubated at 37°C for 30 min with 0 to 200 nM PMSF. The residual activity was determined by the PDAB method. The initial activity of the PA preparations obtained from the wild and the mutant strain was adjusted to 1.2 U/ml.

Effect of pH on PA activity and stability

The activity was measured by PDAB method at different pH values: 5, 6 (acetate buffer 0.1 M), 8 (phosphate buffer 0.1 M) and 10 (carbonate buffer 0.1 M), at 37°C. pH stability profiles of PA were obtained for the wild type and mutant strain of *E. coli* by incubation of the enzyme at 37°C in the same buffers. The residual activity was measured at pH 7.8 in phosphate buffer by the PDAB method, at various times.

Fed batch fermentation.

PA-M1-strain was produced by fed batch fermentation (O.T. Ramírez, et al., to be published elsewhere). The PA activity obtained was 0.18 U/mg prot.

RESULTS AND DISCUSION

Design of mutagenesis regime.

Based on the analysis of the sequence of the PAEC gene (20), several regions were selected that offered the best chances of being in the surface and would not interfere with strongly predicted secondary structure or important residues previously identified(6,9,15,22,27), see Fig. 2. Mutagenic oligonucleotides were synthesized that incorporated variability within the selected sequences. A mixture of bases was employed that afforded codons favoring the creation of possitively charged residues and avoided negatively charged ones (design to be published elsewhere).

Mutagenesis of PAEC gene.

Colonies of the resulting libraries were transfered to filter paper and replicated to a kanamycin containing plate for subsequent recovery. Protein was fixed by chloroform vapor and the filter papers where then subjected to a pH 10 solution, at room temp. for 3 hours, and finally assayed for color due to the presence of 6-APA. Under these conditions, no color was detected at the location of colonies bearing the wild type enzyme. Out of a total of 1500 colonies scored, two showed persistance of color and were designated M1 and M3. To verify the identity of the mutants, restriction fragments corresponding to the region targeted for mutagenesis were subcloned into the wild type gene, substituting the appropriate DNA fragment. The phenotypes of colonies bearing these subcloned fragments were verified and retained the altered pH stability under the assay conditions.

Plasmid DNA was sequenced for the entire subcloned fragment of mutant M1, which was originated in the experiment targeting the beta subunit. The sequence of the mutant revealed an alteration at amino acid 431, Arg for Trp. Mutant M3 originated from the experiment targeting subunit alpha, and its sequence has not been established yet. We describe here the characterization of mutant M1.

Characterization of the engineered enzyme.

Although PA presents a complex kinetic behaviour, due to both products and substrate inhibition, initial rate experiments at low penicillin concentrations Michaelis-Menten kinetics were followed. Therefore the kinetic parameters, Km and k_{cat} were determined directly from assays where the initial rates were measured at various substrate concentrations. Whole cells were used directly for the assays of PA activity as there are no diffusional barriers, side reactions or interference. This is shown by the fact that the Km values for the whole cells and the purified enzyme are of the same order of magnitude (results not shown, see also (21,24)).

In Fig. 3 the pH-activity profile is shown for both the native enzyme and the mutant M1. We observed no significant differences in the profiles, with both enzymes showing an optimum pH for activity between 7 to 8 at 37°C. This demostrates that the strategy of PA modification, suceeded in producing a mutant which did not interfere with important aminoacids related with the secondary structure or the active site.

The molar concentration of PA was determined by reaction with PMSF, a competitive inhibitor (26). After 30 min incubation with the inhibitor the PA activity was assayed. These results are shown in Fig. 4 for enzyme solutions containing the same activity of the native and the mutant PA (1.2 U/ml), using various concentrations of inhibitor. It is shown that both enzymes have the same specific activity, obtained by extrapolation of the straight line. The k_{cat} value in both cases is 5500 min^{-1}. In initial rate experiments it was also found that both enzymes have the same Km value of 12 mM, allowing to conclude that both enzymes are similar in their catalytic behaviour and therefore in the structure of the active site.

In order to analyze the behaviour of the modified penicillin acylase in terms of stability to acidic and basic conditions, both enzymes were stored at 37°C at pH 5, 6, 8 and 10 and subsequently assayed. The results are shown in Fig. 5, where it may be observed that, at acidic pH values, there are no significant differences in behaviour between the two enzymes, while at pH 10 there is a rapid deactivation of both the wild and the modified PA. However, there is a sustantial increase in stability of the modifed enzyme shown by the results obtained at pH 8. In general first order deactivation kinetics are followed: in table 1, the half life of both enzymes at the various pH values are reported. The enzymes show similar stabilities at pH 6 with no activity lost during the experiment (50 h). They also deactivate at the same rate at pH10 and there is a small diference at pH 5. Nevertheless there is a striking difference at pH 8, where the mutant protein stability is increased almost two fold (the

half-life of engineered penicillin acylase is increased from 77.29 h to 145 h). As the mutant enzyme is also less stable at pH 5, there seems to be a shift of the pH-stability profile towards the alkaline side. This is expected from an enzyme where a modification in the number of charged amino acids on the surface would result in a modification of the isoelectric point. In short, the mutant is not altered on its kinetic properties but the stability towards alkaline conditions is improved.

CONCLUSION

It has been previously shown that Protein Engineering is a tool to modify many enzyme properties related with the kinetic and stability behavior. In this paper we demonstrate that minor modifications of the sequence of PA result in mutants with higher stability at non-neutral pH.

Up to now very stable PA derivatives had been obtained by different immobilization techniques so the biocatalyst can stand thousands of hours without considerable activity loses caused by thermal deactivation, as shown by storage stability experiments. Another instability factor affecting the activity is pH shifts during operation. The enzyme obtained by protein engineering may be stabilized by the same immobilization procedures than the wild enzyme as the modification in the structure is minor and does not involve amino acids participating in covalent linkages between the enzyme and the classical supports. It has however the advantage of new pH-stability properties that will be expected to contribute to a higher operating life of the biocatalyst. This strategy is also useful for enzymes whose activity is a function of pH. This is the case of PA, where the forward and reverse reactions differ in optimal pH.

Aknowledgements:

We are grateful to Fernando González for technical support. This work was funded by a grant from PAPIID, UNAM.

Table II Half-life at different pH values for the native PA and the mutant PA named M1

	t1/2 (h)	
	nature PA	Mutant PA-M1
pH 5	135	103.5
pH 6*	---	---
pH 8	77.29	145
pH 10	0.025	0.025

*Activity was constant for 50 h.

Figure leyends

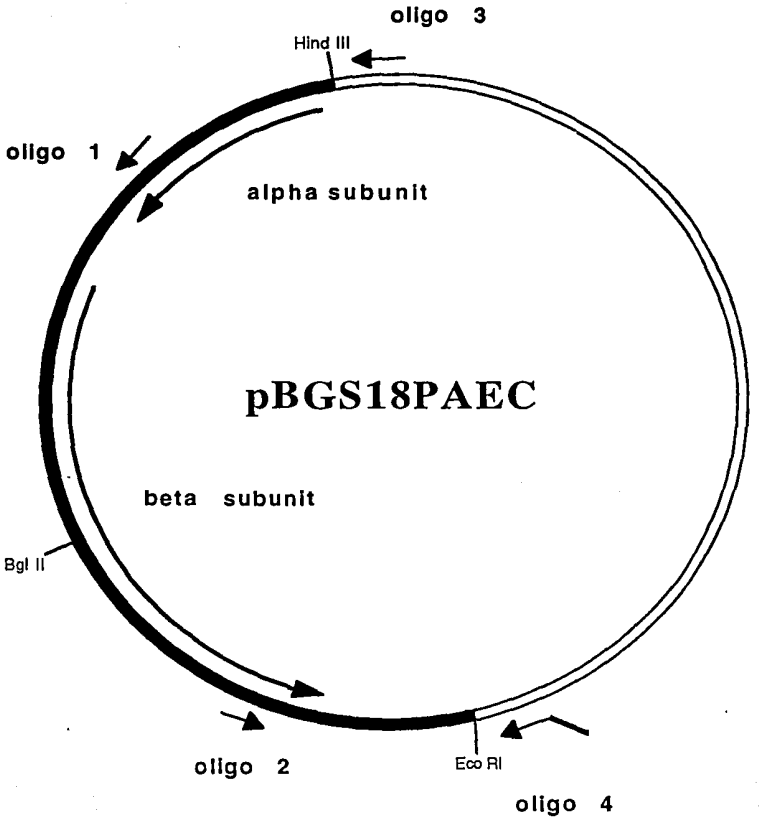
FIG. 1.- pBGS18PAEC MAP.- The original plasmid expressing PAEC gene is pBGS19PAEC (a hindIII SmaI fragment from pP42 CLONED INTO Pbg518 (30); PCR template was pBGS18PAEC, obtained from the former (with the *EcoRI*-HindIII fragment in the opposite orientation with respect to the lac promoter). The figure shows the oligos employed for PCR mutagenesis and also the restriction sites for subcloning the mutagenized fragment from alpha and beta subunit (see Material and Methods).

FIG. 2.- PAC ALIGNMENTS.- A) The alpha (A) and beta (B) subunit for each PAC reported in the GenBank database are aligned against the PAEC sequence. Strongly predicted Alpha -helix (hh) and hidrophylicity (--) regions, as well as the exential residues (*) and the target region for mutagenesis (↓), are marked.

FIG. 3.- pH ACTIVITY PROFILES.- pH activity profiles for PAEC ( ) and M1 ( ) strains are shown. Averages of at least three separate measurments are used.

FIG. 4.- ACTIVE SITE TITRATION.- The molar concentration for PAEC ( ) and M1 ( ) proteins were determined by the active site titration with PMSF (see Materials and Methods). Average of at least three independent measurments.

FIG. 5.- pH STABILITY.- Storage stability of the PAEC ( ) and M1 ( ) protein were


oligo 3
Hind III
oligo 1
alpha subunit
pBGS18PAEC
beta subunit
Bgl II
oligo 2
Eco RI
oligo 4

(A) Penicillin acylase from *E. coli*, alpha subunit - 210 aa
(B) Penicillin acylase from *K. cytrophila*, alpha subunit - 209 aa
(C) Cephalosporin acylase from *Pseudomonas sp.* (acyI), alpha subunit - 238 aa
(D) Cephalosporin acylase from *Pseudomonas sp.* (acyII), alpha subunit - 169 aa

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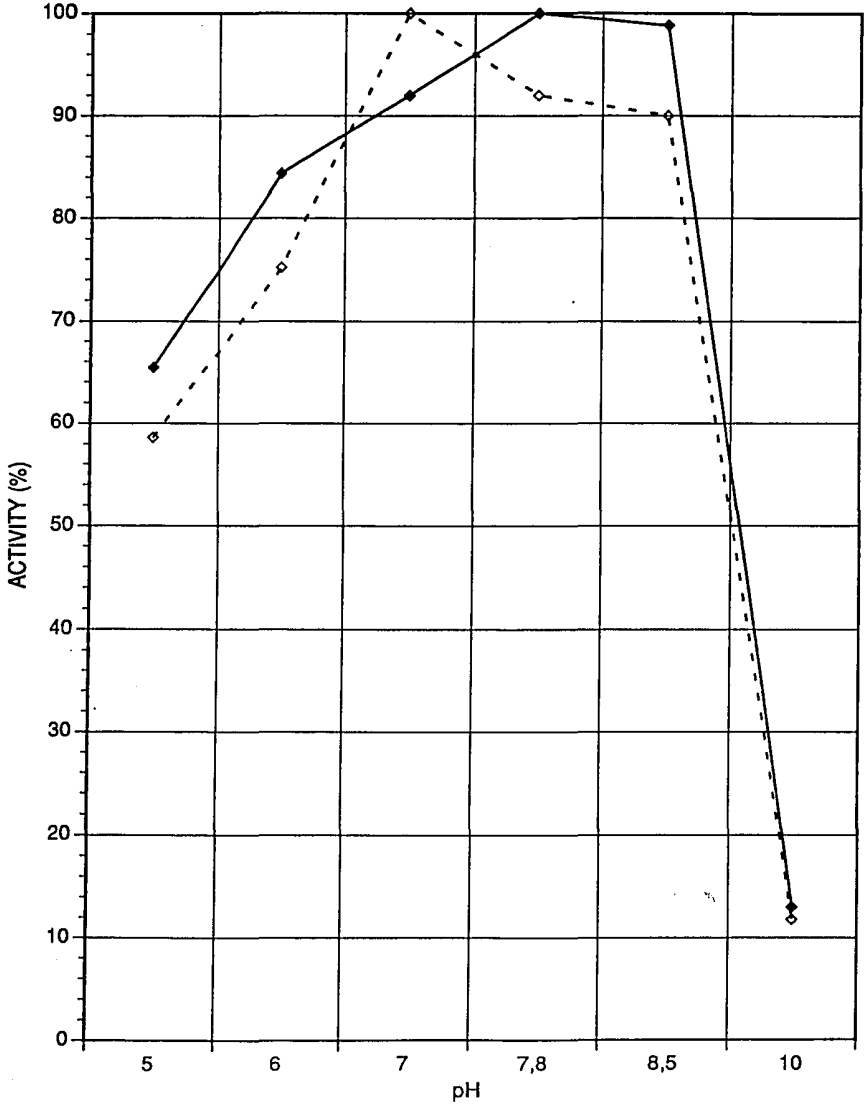
          10        20        30        40        50        60

(A)  SSSEIKIVRDEYGMPHIYANDTWHLFYGYGYVVAQDRLFQMEMARRSTQGTVAEVLGKDF
(B)  PPTEVKIVRDEYGMPHIYADDTYRLFYGYGYVVAQDRLFQMEMARRSTQGTVSEVLGKAF
(C)  .SAPVRVQRDGWGIPHIKASGEADAYRALGFVHAQDRLFQMELTRRKALGRAAEWLGAEA
(D)  ......ILWDGYGVPHIYGVDAPSAFYGYGWAQARSQGDNILRLYGEARGKGAEYWGPDY

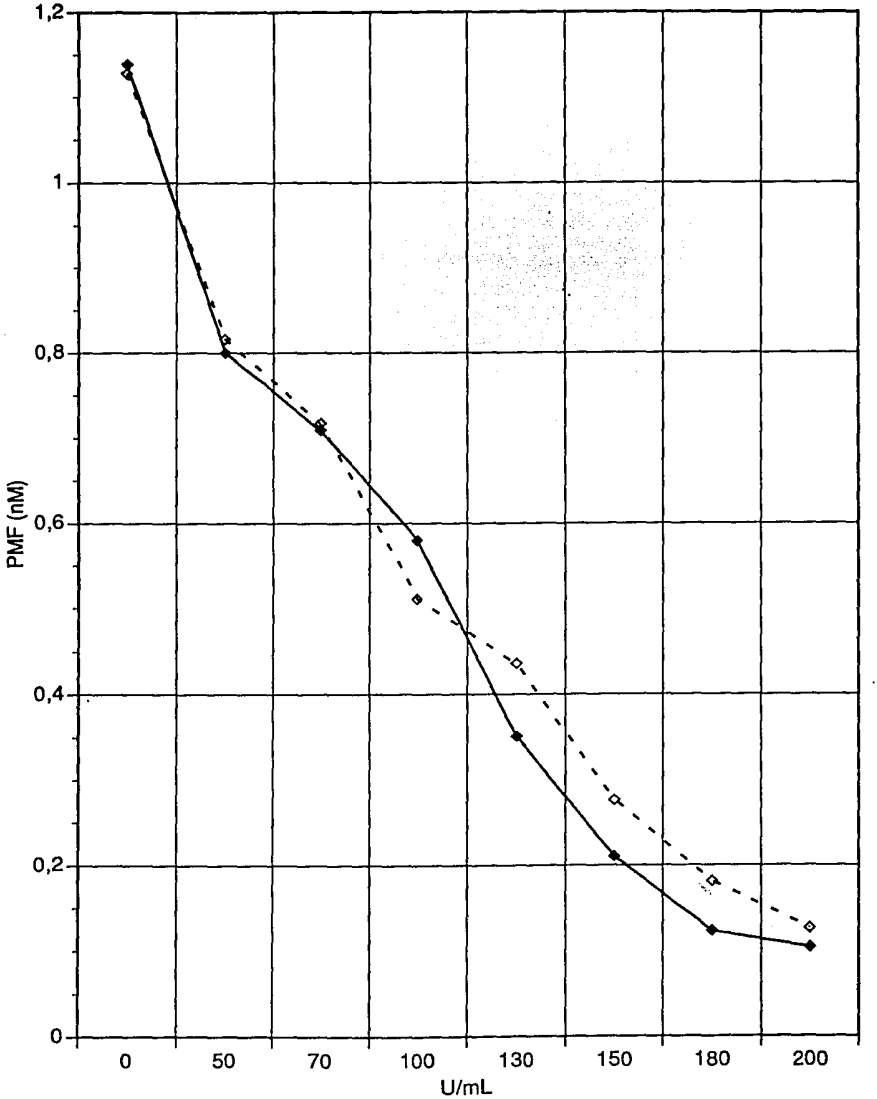
          70        80        90       100       110       120
         ------------------
           hhhhhhhh
(A)  VKFDKDIRRNYWPDAIRAQIAALSPEDMSILQGYADGMNAWIDKVNTNPETLLPKQFNTF
(B)  VSFDKDIRQNYWPDSIRAQIASLSAEDKSILQGYADGMNAWIDKVNASPDKLLPQQFSTF
(C)  AEADILVRRLGMEKVCRRDFEALGAEAKDMLRAYVAGVNAFL-----ASGAPLPIEYGLL
(D)  EQTTVWLLTNGVPERAQQWYAQQSPDFRANLDAFAAGINAYAQQ

                                                    ⬇
      130       140       150       160       170       180
     ----------  * *                  ---------
(A)  GFTPKRWEPFDVAMIFVGTMANRFSDSTSEIDNLALLTALKDKYGVSQGMAVFNQLKWLV
(B)  GFKPKHWEPFDVAMIFVGTMANRFSDSTSEIDNLALLTAVKDKYGNDEGMAVFNQLKWLV
(C)  GAEPEPWEPWHSIAV
```

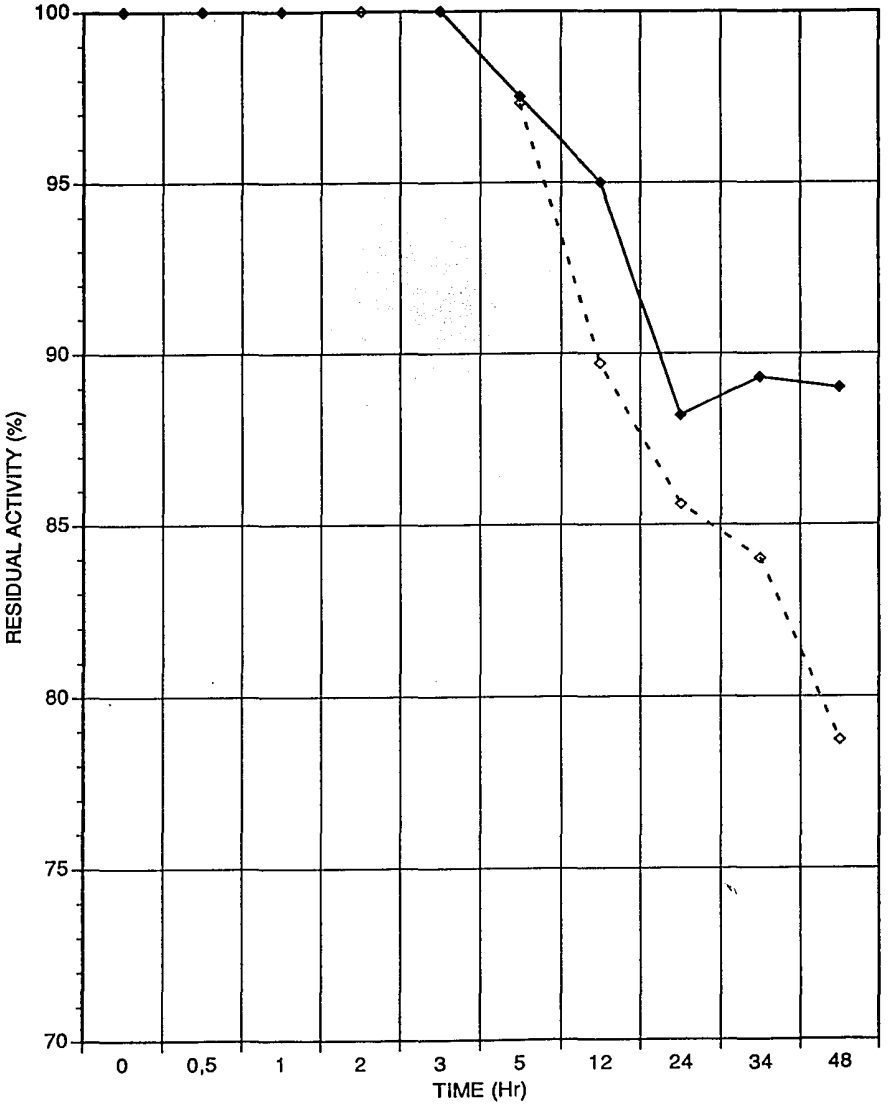
3



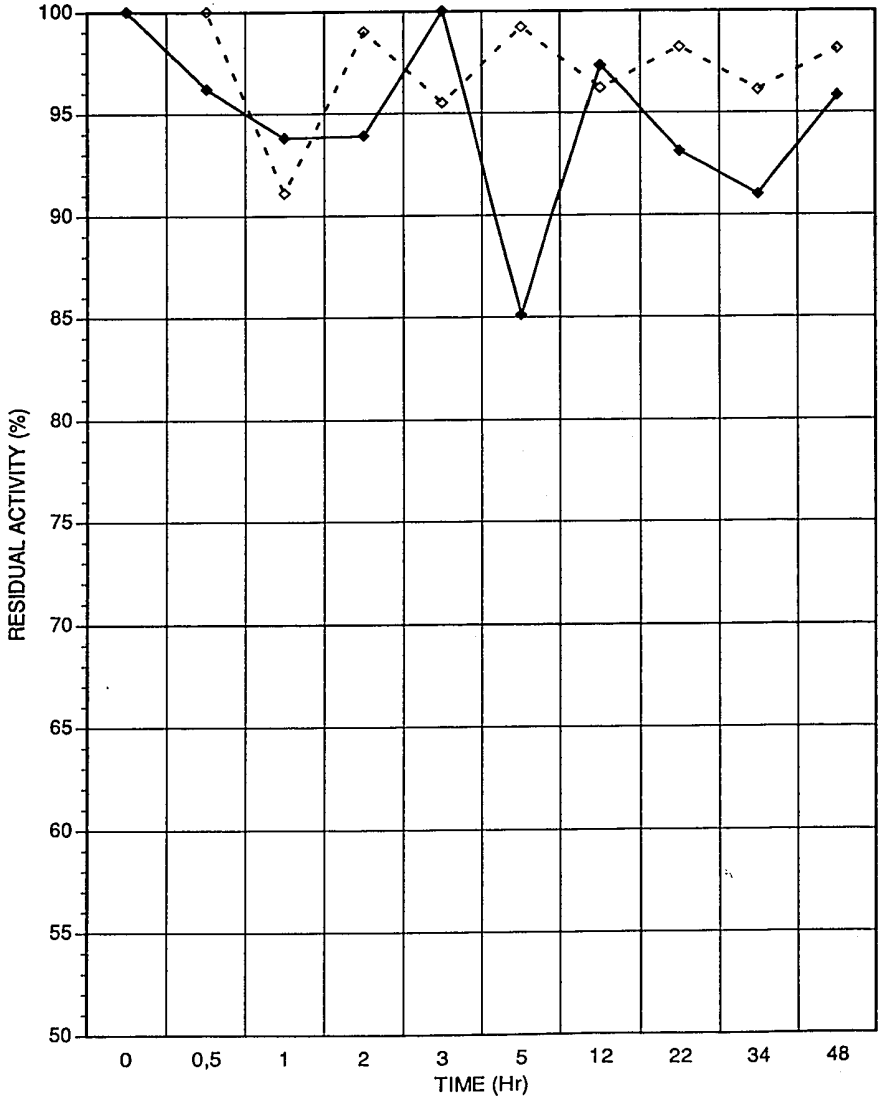
4



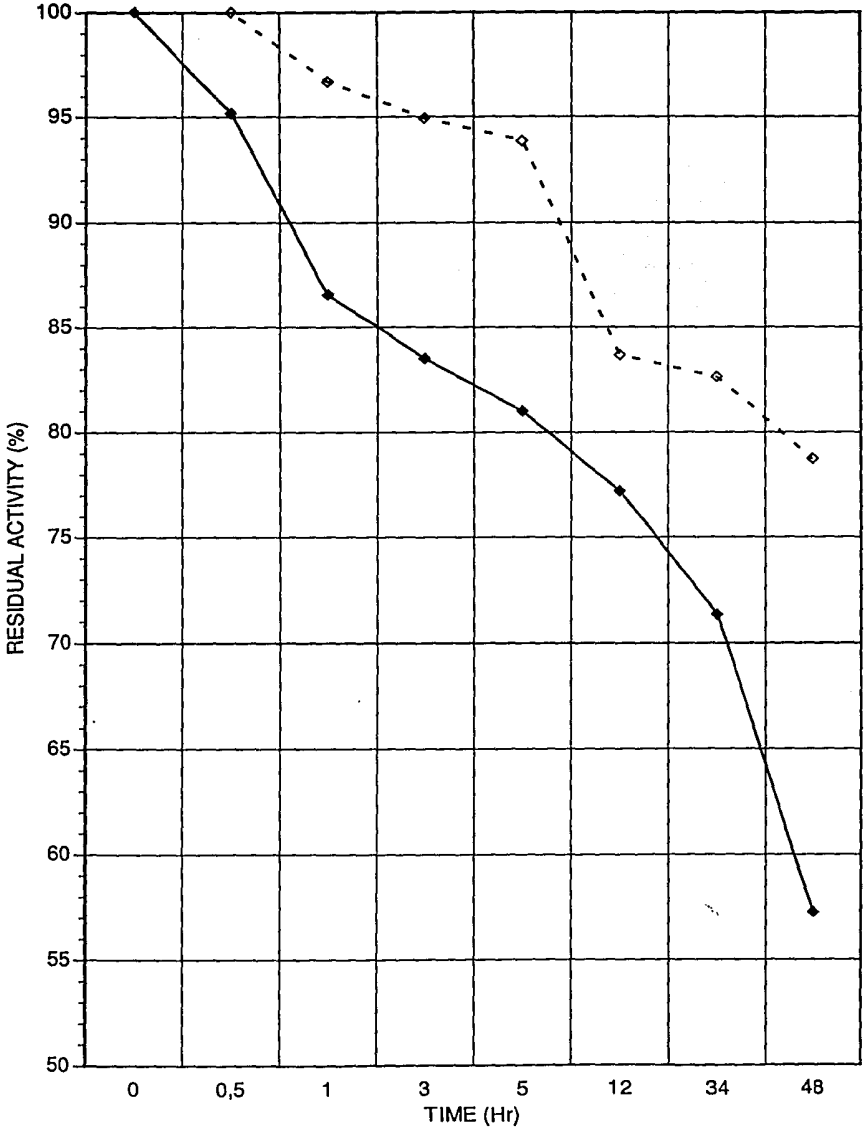
A


100
95
90
85
80
75
70
RESIDUAL ACTIVITY (%)
0
0,5
1
2
3
5
12
24
34
48
TIME (Hr)

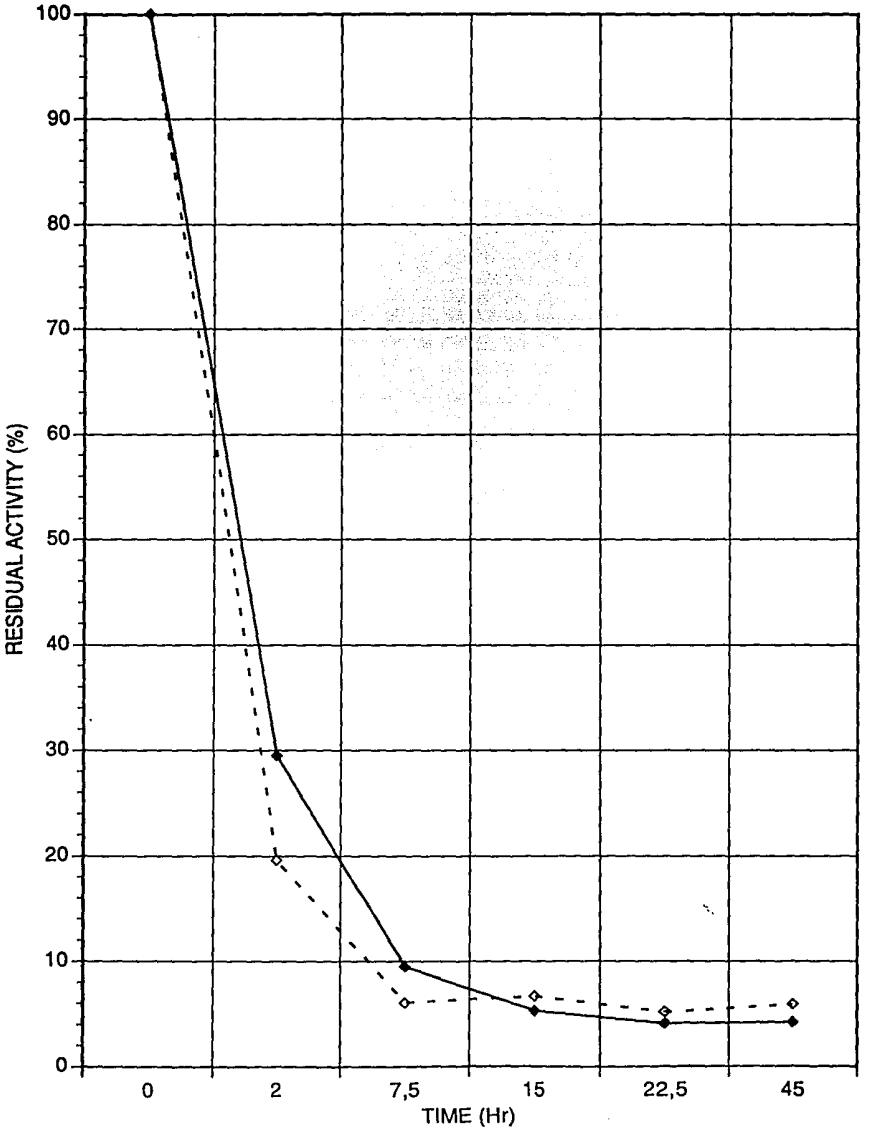
B


100
95
90
85
80
75
70
65
60
55
50
RESIDUAL ACTIVITY (%)
0
0,5
1
2
3
5
12
22
34
48
TIME (Hr)

C


100
95
90
85
80
75
70
65
60
55
50
RESIDUAL ACTIVITY (%)
0
0,5
1
3
5
12
34
48
TIME (Hr)

D


100
90
80
70
60
50
40
30
20
10
0
RESIDUAL ACTIVITY (%)
0
2
7,5
15
22,5
45
TIME (Hr)

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